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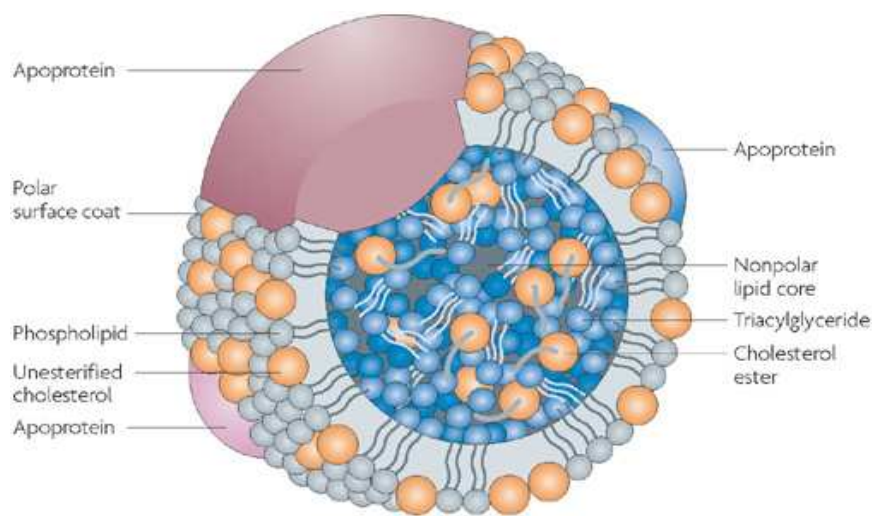
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1 INTRODUCTION

1.1 Lipid Metabolism

Lipids are a diverse group of compounds, for example triacylglycerides, cholesterol, phospholipids, vitamins A,D,E,K and many other molecules (3), which are essential for diverse biological functions, such as maintaining cell membrane structure and function, oxidative metabolism via β -oxidation, cell signalling, hormone synthesis and many others. To serve all these purposes, lipids need to be transported to those tissues and cells which require exogenous lipids. As the bloodsystem of all animals is aqueous, plasma lipids are transported in form of water-soluble lipoproteins (Fig 1.1.1). Lipoproteins are spherical complex macromolecules which consist of an hydrophobic inner core, made up of cholesteryl-esters (CE) and triacylglycerides and a hydrophilic outer surface layer composed of phospholipids, unesterified cholesterol and apoproteins (3).



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Fig.1.1.1 Lipoprotein Structure.

(www.nature.com/nrd/journal/v7/n1/images/nrd2353-f1.jpg)

Processing of lipoproteins in the body is called lipoprotein metabolism (Fig 1.1.2), and can be divided into two distinct pathways.

1. The exogenous pathway, in which dietary lipids and those secreted into the intestinal lumen via bile, are taken up by epithelial cells of the small intestine and then get secreted in form of chylomicrons into lymph and finally enter the bloodstream via the thoracic duct. Lipoprotein lipase (LPL) catalyzes the hydrolysis of triacylglycerol, releasing glycerol and free fatty acids (FFA), which then can be absorbed by peripheral tissues. Hydrolyzed chylomicrons (CM) are called chylomicron remnants and are rapidly taken up by the liver(3).
2. The endogenous pathway, in which lipoproteins are synthesized by the liver. Lipids are secreted into blood by the liver in form of very low-density lipoproteins (VLDL), which circulate through the body and thereby get hydrolyzed by LPL, again resulting in glycerol and FFAs being absorbed by peripheral tissues. Thereby VLDL particles develop into intermediate-density lipoproteins (IDL) which can be taken up by the liver or be further hydrolyzed by hepatic lipase, leading to absorption of glycerol and FFAs by the liver. So called low-density lipoproteins (LDL), the cholesterol-rich remnants of VLDL, are taken up either by peripheral tissues or the liver. Another class of lipoproteins, high-density lipoprotein (HDL), mediates reverse cholesterol transport from peripheral tissues to the liver, where cholesterol either is converted into bile or repackaged into VLDL particles. (3)

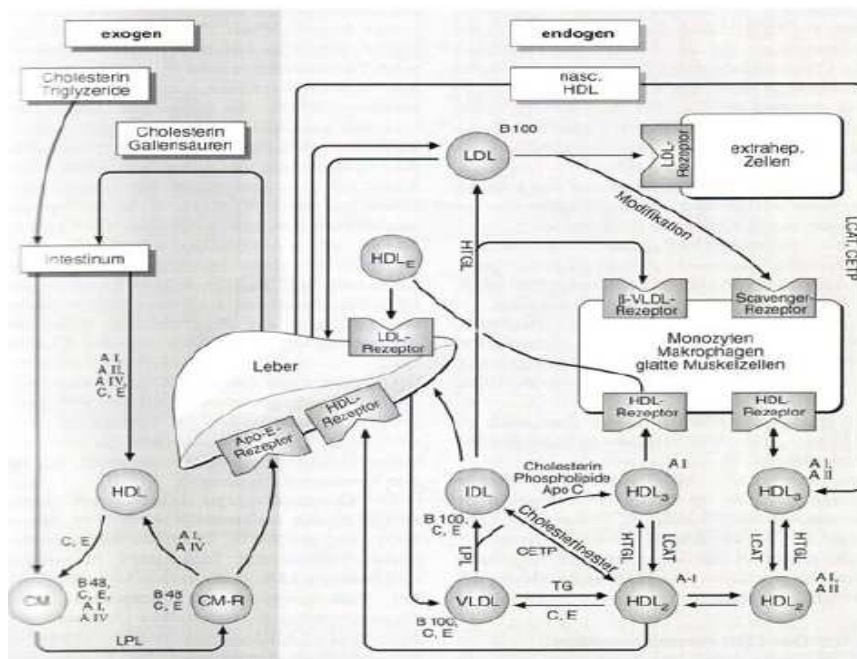


Fig 1.1.2 Lipoprotein metabolism

(www.univie.ac.at/Medbch/MolMed1/Hermannklein08.pdf)

1.2 Lipoproteins

Distinct subtypes of lipoproteins differ in size, lipid-, apoprotein composition, and density. The latter characteristic is the major basis for classification of lipoproteins. Each class of lipoproteins has a specific role in lipoprotein metabolism as already described in the chapter above (3).

Name	Density [g/ml]	Diameter [nm]	Major Apo-protein	Major Lipid Component[%]	Sources
CM	<0.95	100-1000	ApoB48/ ApoE	Triacylglyceride 84	Intestine
VLDL	0.95-1.006	30-80	ApoB100/ ApoE	Triacylglyceride 50	Liver
IDL	1.006-1.019	25-50	ApoB100/ ApoE	Triacylglyceride 31	Hydrolyzation of VLDL
LDL	1.019-1.063	18-28	ApoB100	Cholesterol 50	Hydrolyzation of IDL
HDL	>1.063	5-15	ApoA1/II	Cholesterol 30	Liver, Intes- tines

Table 1.2 Classification of lipoproteins

1.3 Apolipoproteins

Apolipoproteins, also called apoproteins, are the protein components of lipoproteins and play key roles in lipoprotein metabolism:

1. Stabilization of lipoproteins
2. Together with phospholipids, generation of a hydrophilic surface
3. Essential for synthesis and secretion of lipoproteins
4. Transportation of lipids
5. Binding to specific cell-surface receptors
6. Decisive for lipid transfer from lipoproteins to cells or vice versa.
7. Cofactors for specific enzymes, like Lecithin-Cholesterol Acyltransferase (LCAT)

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Apoproteins can be divided into classes and subtypes (Table 1.3).

The most important are:

1. Apoproteins A (Subtypes: apoA-I, apoA-II, apoA-IV): first found in HDL
2. Apoproteins B (Subtypes: apoB-48, apoB-100): first found in LDL
3. Apoproteins C (Subtypes: apoC-I, apoC-II, apoC-III) : first found in VLDL
4. Apoprotein E (three common isoforms exist : E-1, E-2, E-3, which differ in the amino acids at position 112 and 158, and in their functionality)

Each apoprotein has a specific function and is part of specific lipoproteins. Nonetheless, apoproteins can be and in fact are often exchanged between lipoprotein particles of different classes, like the transfer of apoC and apoE from HDL to CMs for example.

Name	Molecular Weight [kDa]	Lipoproteins	Function
ApoA-I	28.5	CM, HDL	Activation of LCAT, Binding of SRBI (HDL receptor), selective lipid uptake, reverse cholesterol transport
ApoA-II	17	CM, HDL	Activation of hepatic Lipase
ApoA-IV	46	CM, HDL	Activation of LCAT
ApoB-48	265	CM	Structural element
ApoB-100	550	VLDL, IDL, LDL	Binding to LDL receptor, endocytosis
ApoC-I	6.5	CM, VLDL, IDL	Activator of LCAT
ApoC-II	8.8	CM, VLDL, IDL	Activation of LPL
ApoC-III	8.9	CM, VLDL, IDL	Inhibition of LPL
ApoE	34	CM, VLDL, IDL, HDL	Binding to apoB/E receptors endocytosis, reverse cholesterol transport

Table 1.3 Apoproteins

1.4 Cholesterol Metabolism

Cholesterol is a polycyclic alcohol, that belongs to the lipid class of steroids and is essential for many crucial biological functions (3).

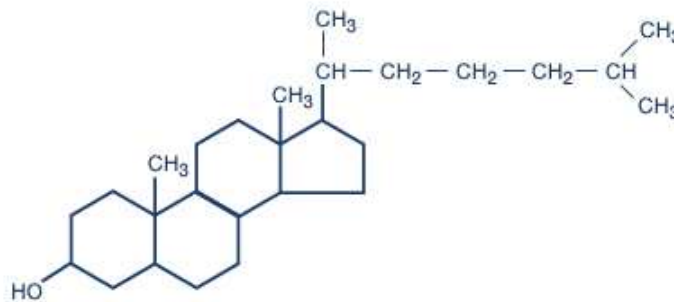


Fig.1.1.4. Structure of Cholesterol

(www.Lipid-liga.de/cms/images/stories/cholesterin.jpg)

In addition to the housekeeping functions of cholesterol in all mammalian cells as a key contributor to membrane structure and function, it is the precursor for cytochrome P450 cholesterol side chain cleavage enzyme-initiated synthesis of steroid hormones. Cholesterol can also be converted to bile acids in the liver and to vitamin D in the skin and kidney (3). Therefore, multiple pathways have evolved to ensure full supply of animal cells with cholesterol, especially in steroidogenic tissues and the liver (3):

1. Endogenous synthesis from acetyl coenzyme A (acetyl CoA)
2. Hydrolysis of cholesteryl esters stored in cytoplasmic lipid droplets
3. Exogenous cholesterol transported to the cells by lipoproteins and taken up via cell surface receptor binding, followed by either endocytosis of the lipoprotein particle or selective lipid uptake without endocytosis (This will be the topic of later chapters of this introduction).

The homeostasis of cholesterol synthesized endogenously and taken up from the diet is regulated by several mechanisms, especially via regulation of 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase, which catalyzes the rate-limiting step in cholesterol synthesis, the reduction of 3-hydroxy-3-methylglutaryl-coenzyme-A to mevalonate. Homeostasis of cholesterol is crucial, as it is essential for living, but on the

other hand, high plasma levels of cholesterol (LDL-cholesterol) correlate with atherosclerosis and coronary artery disease. Therefore cholesterol metabolism, as well as its regulation is a highly interesting research topic and its participating molecules are promising targets for novel therapeutic approaches (3).

1.5 Cell Surface Receptors

As already described in the chapter above, 2 different pathways exist for cellular uptake of cholesterol from lipoproteins (3).

1. Endocytotic uptake of LDL particles, mediated by the LDL receptor (LDLR) or LDLR relatives (LR), proteins with a high degree of structural similarity to LDLR (6). These receptors form the so-called LDLR supergene family
2. Selective lipid uptake from HDL, mediated by scavenger receptors class B type I and type II (SRBI and SRBII), a process which does not involve endocytosis of the lipoprotein particle.

1.5.1 The LDLR Supergene Family

LDLR (see Fig 1.5.1) is built up of 7 head-to-tail arranged type A binding repeats (LA repeats) of ~40 residues, harboring six paired cysteines in identical positions; 3 type B repeats also containing six cysteines each, modules of ~50 residues with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD); six of these modules form a so-called beta propeller, which together with the type B repeats constitutes the epidermal growth factor precursor (EGFP) homology domain; a short stretch rich in serines and threonines carrying O-linked sugars (the so called O-linked sugar domain); a single transmembrane domain of approximately 20 amino acids; and the C-terminal cytoplasmic region with a short signal for receptor internalization (NPxY) (6).

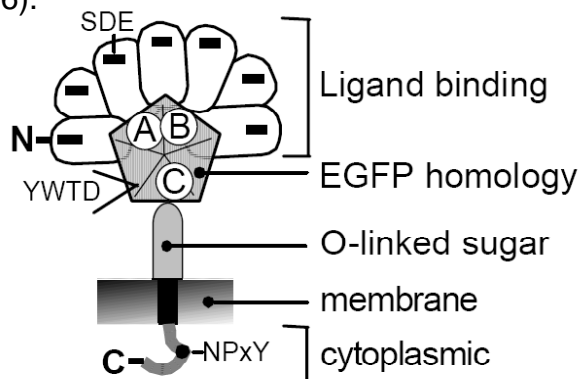


Fig.1.5.1 Structure of LDLR

www.univie.ac.at/Medbch/MolMed1/Schneider2008.pdf

Other proteins of the LDLR supergene family (Fig 1.5.2) are of the same modular basis, but differ in size, number or arrangement of modules and additional domains. Prominent members are LDL receptor related protein (LRP1), LRP2, VLDL receptor (VLDLR), LDLR relative 11 (LR11/sorLA), ApoE receptor type 2 (ApoER2), LR7/8B, LRP 3,4,5,6 and LRP1B (6).

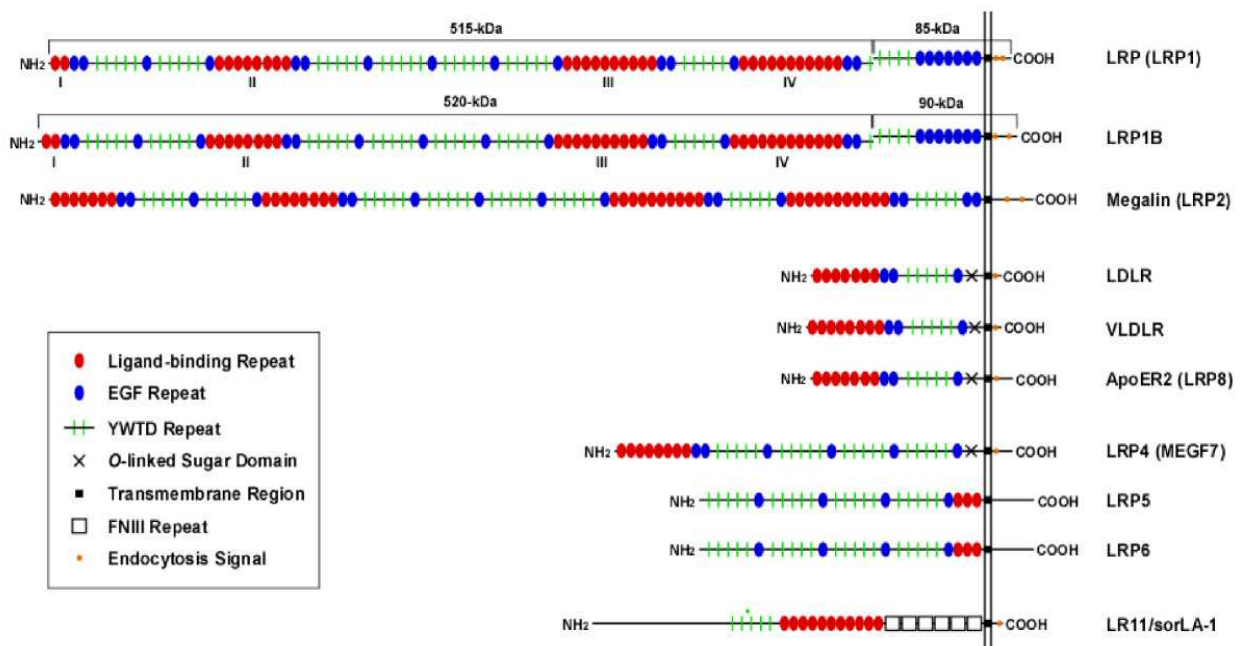


Fig 1.5.2 The LDLR supergene family

(www.molecularneurodegeneration.com/content/figures/1750-1326-1-8-2l.jpg)

LDLR is found in most cell types and assembles as cell surface receptor in clathrin-coated pits of the plasma membrane of animal cells. LDLR binds to apoB-100 in LDL particles or to apoE in VLDL and chylomicron remnants, and thereby mediates internalization of the bound particles via clathrin-coated vesicles and subsequently, endosomes. LDLRs are recycled back to the plasma membrane, whereas the endosomes fuse with lysosomes, where the LDL particle gets degraded. Cholesterol can then be further utilized or esterified and stored in a cholesterol ester lipid droplet (3).

1.5.2 Scavenger Receptors

Scavenger receptors are defined as cell surface membrane proteins that bind chemically modified lipoproteins such as acetylated LDL, oxidized LDL, and many

other types of ligands (1) like sulfated polysaccharides or polynucleotides. Because of their broad binding specificity, these receptors have been named scavenger receptors. Since the first scavenger receptor has been identified in 1994 by M. Krieger and J. Herz, cDNAs of many distinct scavenger receptors have been cloned and analyzed, from organisms as diverse as mammals, avians, fish, or fruit flies. These receptors have been categorized into broad classes (A,B,C,...) based on global structure similarities. In many cases, the members of a given class have been subdivided into “types” based on more subtle structural differences, including multiple proteins from a single gene generated by alternative RNA splicing (1). In my work I focused on scavenger receptor class B type I (SRBI) and its isoform SRBII.

1.6 Scavenger Receptor Class B Type I

SRBI was first identified in 1994 by Endemann, Acton, Krieger and colleagues in a scavenger receptor expression study that used acetylated LDL as ligand. Like other scavenger receptors, besides acetylated LDL, SRBI binds a broad class of ligands, like oxidized LDL, maleylated BSA, anionic phospholipids, apoptotic cells, unmodified LDL and VLDL and, with high affinity, HDL. SRBI therefore was the first identified HDL receptor of physiological relevance (1).

1.6.1 Structure of SRBI

SRBI is a member of a family of structurally related proteins, called the CD36 superfamily, which also includes the mammalian proteins CD36 and LIMPII, D. melanogaster proteins emp and croquemort, SnmP-1 and others (3).

Human SRBI is a protein of 509 amino acid length, with a horseshoe like topology, consisting of a large extracellular loop (403 amino acids) anchored to the plasma membrane by N- and C-terminal transmembrane domains (28- and 25 amino acids), which have short extensions into the cytoplasm (8 N-terminal- and 45 C-terminal amino acids) (1). 8 cysteines are found within the protein, 6 of them are located in the loop region, one in the C-terminal transmembrane- and one in the C-terminal cytoplasmic domain. The transmembrane (position 426) and cytoplasmic (position 470) cysteines are palmitoylated (1). SRBI is heavily N-glycosylated, and therefore its molecular weight predicted from its amino acid sequence of ~57kDa differs clearly

from the observed ~82kDa using immunological methods. In cultured cells, SRBI, just like its isoform SRBII, clusters in caveolae-like lipid domains, which are clathrin-independent small invaginations of the plasma membrane rich in protein and cholesterol. The mechanism by which the subcellular localization of SRBI is regulated is not yet known (1).

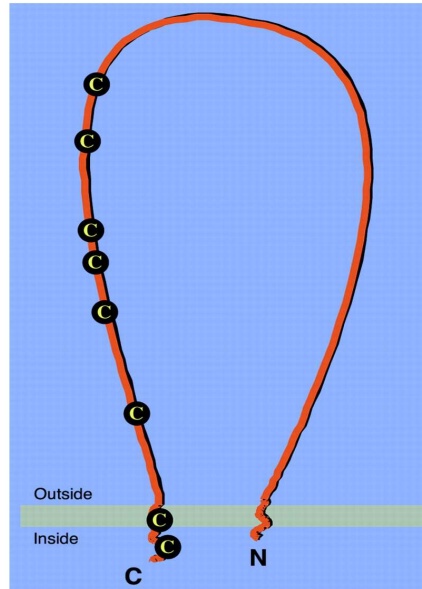


Fig.1.6.1 Structure of human SRBI

www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=11560945

1.6.2 Function of SRBI

SRBI facilitates lipid transfer, mainly in form of cholesteryl esters, but also of others like phospholipids or α -tocopherol, between different cell types of animals and lipoproteins. The main function of SRBI is binding HDL and thereby mediating transfer of cholesteryl esters in both directions, either from the hydrophic core of the HDL particle into the cell, or the reverse way from the cell to HDL. This mechanism neither involves internalization nor lysosomal degradation of the lipoprotein particle and is essential for providing adequate amounts of cholesterol to extrahepatic cells, especially those of steroidogenic tissues and for secretion of plasma cholesterol via the bile (1,3).

Hepatic overexpression of SRBI in mice leads to a decrease in plasma HDL cholesterol- and apoA-I concentration, as well as to an increase in hepatic bile cholesterol concentration (1,3).

Compared to wild type mice, SRBI knock-out mice, show a 2-2.5 times elevated total plasma cholesterol concentration caused by an increase in large HDL particles heterozygous in size, which are abnormally enriched in apoE. Plasma apoA-I concentration does not change, indicating that impaired selective lipid uptake from HDL-CEs is the reason for the increase in total plasma cholesterol levels. The SRBI knock-out also decreases biliary- and gallbladder bile cholesterol content, without altering bile acid or phospholipid secretion, bile acid poolsize and fecal bile excretion. These findings indicate that SRBI is essential for selective lipid uptake and reverse cholesterol transport. Therefore, SRBI plays a key role in controlling the structure and amount of plasma HDL cholesterol, steroidogenic and hepatic uptake of HDL cholesterol, and the use of HDL cholesterol for biliary cholesterol secretion (1,3).

Net flux of lipids is likely driven by the concentration gradient between cells and extracellular donor/acceptor particles (3). Hence enzymatic and non- enzymatic modulation of HDL lipid composition may influence net flux of lipids mediated by SRBI (3).

Selective Lipid Uptake

Cellular uptake of HDL-cholesteryl esters mediated by SRBI is called selective lipid uptake. This process appears to be a two-step process, consisting of (1,3):

1. High-affinity lipoprotein binding to the receptor
2. Receptor mediated transfer of cholesteryl esters

Selective lipid uptake mediated by SRBI is followed by dissociation of the lipid depleted lipoprotein from the receptor (1,3).

SRBI also binds and facilitates selective lipid uptake from LDL and VLDL particles, but significantly less efficient than compared with HDL-SRBI interaction. HDL competes effectively for LDL binding to SRBI, whereas LDL can only partially compete for HDL binding to SRBI (1), a mechanism called nonreciprocal cross-competition (NRCC). This indicates that SRBI probably has multiple binding sites for different ligands, with different ligand binding properties. The molecular basis for this mechanism is not yet understood (1,3).

High affinity binding of HDL is mediated by SRBI as well as by CD36 (its homolog), but in contrast to SRBI, CD36 is not able to facilitate selective lipid uptake. Analysis of SRBI/CD36 chimeras established that sequences in the extracellular domain of SRBI are sufficient to confer efficient selective lipid uptake activity on the cytoplasmic and transmembrane domains of CD36, indicating that the extracellular loop of SRBI is essential for lipid transfer (3). On the other hand, the carboxy-terminal domain of SRBI can influence efficiency of selective lipid uptake, as SRBI facilitates this process significantly less efficient, than its isoform SRBI (2,3). Furthermore SRBI ligand binding and selective lipid uptake depend on the apolipoproteins present in the lipoprotein particle. In case of HDL, apoA-I and apoA-II are the major apoprotein components. ApoA-I deficient HDL binds SRBI with high affinity, but no lipid transfer occurs (3). ApoA-I and apoA-II composition of HDL particles also seem to influence the interaction between the lipoprotein and its receptor. ApoA-II enrichment of HDL results in an increased binding affinity for SRBI, but inhibits selective lipid uptake. Reconstituted apoA-I/apoA-II-containing particles compared to single apoA-I containing particles show decreased binding (3). As experiments with reconstituted discoidal apoA-I complexes have shown, either the amino- or the carboxy-terminal amphiphatic helices of apoA-I are necessary for binding of these complexes. These findings suggest that the conformation/organization of apoA-I in HDL particles critically affects the nature of the interaction of the particle with SRBI (3).

As already mentioned above, SRBI clusters in caveolae-like, lipid-rich domains of the plasma membrane in cultured cells, indicating that these domains might have a significant effect on SRBI-mediated lipid transfer (1,3). This hypothesis was supported by the finding, that in certain cells selective lipid uptake seems to correlate with caveolin-1 expression (3). Caveolin-1 is the major contributor to the caveolae scaffold and also has cholesterol binding activity. However, it was shown that caveolin-1 negatively regulates selective lipid uptake facilitated by SRBI and its localization in caveolae-like domains is not necessary for selective lipid uptake. These and further findings have shown that SRBI is able to mediate selective lipid uptake from SRBI independently from specialized cellular structures or compartments (3).

Reverse Cholesterol Transport:

Transport of cholesterol from extrahepatic tissues to the liver, where cholesterol is either secreted into bile or repackaged into VLDL particles, mediated by SRBI in concert with other proteins is called reverse cholesterol transport. The first two findings indicating that SRBI might be involved in reverse cholesterol transport were (3):

1. Caveolae are proposed to be the major sites of cholesterol efflux from cells to lipoprotein particles
2. In different cell lines, expression levels of SRBI correlate with cholesterol efflux to HDL particles

These findings were supported by the phenotype found in SRBI-KO mice and cell culture experiments in which SRBI was blocked either by mutation or with antibodies. Reconstitution of HDL particles with mutant apoA-I forms resulted in decreased cholesterol efflux to these particles. Formation of a productive complex between SRBI and HDL seems to be required for efficient cholesterol transport activity (3).

1.6.3 Regulation of SRBI Gene Expression

To date only little is known about regulation of SRBI expression. The transcription factors steroidogenic factor 1 (SF-1), CCAAT/enhancer-binding protein (C/EBP), SREBP-1, liver X receptor (LXR) and liver receptor homolog 1 (LRH-1) are supposed to play a role in regulation of SRBI gene expression, as consensus-DNA sequences were found within the promotor of the human SRBI gene, which can be bound by these transcription factors and by others yet to be identified. These transcription factors can be stimulated or inhibited by tropic hormones, cholesterol and polyunsaturated fatty acids and other substances (1,3).

SRBI expression is coordinately regulated with cholesterol metabolism (1). In steroidogenic cells, SRBI expression is stimulated by systemic administration of the tropic hormone adrenocorticotrophic hormone (ACTH) via the cAMP/protein kinase A signal transduction pathway, and inhibited by dexamethasone. In testis, SRBI expression is stimulated by human chorionic gonadotropin (3).

High dose estrogen treatment can, in a species-dependent way, either stimulate or inhibit SRBI expression in hepatocytes, steroidogenic cells, and macrophages. The

SRBI expression of these cells can also be altered by peroxisome proliferator-activated receptor (PPAR) agonists, vitamin E, polyunsaturated fatty acids, cholesterol, and other agents (1).

Intestinal SRBI expression is regulated coordinately with cholesterol absorption.

Taking these findings together it appears that SRBI expression is regulated in a species- and cell type-specific way. Thus, versatile regulation systems have evolved.

1.6.4 Tissue Distribution

The highest levels of SRBI expression are found in the liver, which is the central organ for lipid metabolism and in steroidogenic tissues, where high amounts of cholesterol are needed for conversion into steroid hormones (1,3). These tissues will be discussed in more detail in this chapter.

Further SRBI-expressing tissues are the intestine, mammary gland of pregnant rats, trophoblast, yolk sack and placenta, lung, macrophages, endothelial cells, neuroglia, smooth muscle cells, retinal pigmental epithelial cells, and keratinocytes (1,3).

Liver:

The highest total amount of SRBI protein is found in the liver, where 90% of the SRBI-mediated selective lipid uptake of cholesteryl esters from HDL occurs. In rodents lacking cholesterylester transfer protein (CETP), like rats and mice, 50% of total HDL-CE clearance is mediated by the liver, whereas in CETP-expressing animals it is only about 20%. HDL-cholesterol taken up by the liver is either converted into bile or repackaged into VLDL particles (3).

Under basal conditions, hepatic SRBI is mostly expressed on the sinusoidal surface of parenchymal cells. Further hepatic SRBI expression has been reported in liver endothelial cells and Kupffer cells (3). Estrogen treatment of rats resulted in a shift of total liver protein expression from SRBI to SRBII (see point 1.7) and to decreased SRBI expression in parenchymal cells, in contrast to an increase in SRBI expression in Kupffer cells. Chronic ACTH stimulation in rats and mice resulted in a decreased hepatic SRBI expression. It was found that the adrenals, under such circumstances, release a factor repressing hepatic SRBI expression and thereby ensure that enough

Introduction

lipoprotein cholesterol is available (3). These findings strongly suggest that hepatic SRBI expression is sensitive to hormonal regulation. Dietary cholesterol changed SRBI expression patterns in parenchymal and Kupffer cells of rat liver analog to estrogen treatment. No differential expression of liver SRBI, induced by dietary cholesterol, was found in hamsters or mice (3). Thus, hepatic regulation of SRBI by dietary cholesterol seems to be species-dependent. Further substances influencing SRBI expression, reported at least for chinese hamster, are plant-derived polyunsaturated fatty acids, dietary myristic acid, α -tocopherol, LPS, TNF, IL-1, and insulin (3).

Adrenal Gland:

The highest expression levels of SRBI per gram of tissue have been found in rodent adrenal glands (3). The adrenal gland needs high amounts of cholesterol as substrate for synthesis of mineralocorticoids, glucocorticoids, androgens, and small amounts of estrogens, thereby explaining the high concentration of SRBI protein per gram tissue. SRBI is found primarily on the surfaces of steroidogenic parenchymal cells, where it is essential for ACTH-induced microvilli-rich adrenal channel formation (3). These channels are generated by intercellular dimerization of SRBI and serve the purpose of trapping lipoprotein particles. Thus, they are the major site of selective lipid uptake. ACTH induces this process by stimulating SRBI expression, reported for mouse adrenocortical cells in vivo, as well as for cultured human adrenocortical cells. Dexamethasone has a contrary effect and inhibits SRBI expression in adrenocortical cells (3). Estrogen treatment of rats leads to an increase in adrenocortical SRBI expression, followed by enhanced binding of HDL to the adrenocortical cell surface and increased selective uptake of cholesteryl esters from HDL. ACTH seems to be a crucial factor in this process, as hypophysectomy dramatically decreases adrenal SRBI expression, and chronic estrogen therapy affects ACTH secretion (3).

Ovaries:

In the ovaries, SRBI expression patterns depend on the stage of the estrus cycle. During the preovulatory stage, SRBI expression is found only in theca interna cells.

These cells convert cholesterol into androgens and therefore need adequate cholesterol supply (3). In granulosa cells, which convert androgens into estrogens, no SRBI expression occurs. During luteinization, initiated by luteinizing hormone (LH), SRBI expression shifts from theca interna cells to granulosa cells, which then develop into the corpus luteum, synthesizing progesterone and small amounts of estrogen (3). Consistent with that, estrogen administration to rats results in increased SRBI expression, selective lipid uptake, and steroidogenesis in the ovaries. Also, high-dose treatment of rats with estrogen, which resembles the situation before ovulation, shifts SRBI expression from theca interna cells to granulosa cells (3).

Testis:

In Leydig cells, which synthesize androgens, like testosterone essential for spermatogenesis, most of testicular SRBI expression is found, whereas in Sertoli cells, which nurture the developing sperm cells, but also have some other functions like synthesis of the anti-Müllerian hormone during embryogenesis, little SRBI expression occurs (3). Levels of SRBI are very low in general, correlating with very low selective lipid uptake from HDL particles, indicating independence of Leydig cells from exogenous cholesterol sources for steroidogenesis. Consistent with that, no defects in fertility have been detected in male SRBI knockout mice. Yet, gonadotropin increases SRBI expression in Leydig cells, resulting in an increase in SRBI-mediated selective uptake of cholesteryl esters from HDL particles, followed by an increase in steroid hormone synthesis. Thus, it seems like Leydig cells are not dependent on HDL-derived cholesterol, but nonetheless can use it as source (3).

1.6.5 Physiological Role of SRBI

As SRBI is expressed in many and diverse types of tissues and cells, and contributes to many crucial biological functions as mentioned above, it is not surprising that SRBI has been reported to be involved in diverse physiological and pathophysiological processes like atherosclerosis, female fertility, cholesterol gallstone disease, intestinal cholesterol absorption, embryogenesis and fetal development, red blood cell maturation, nitric oxid metabolism, and vitamin E transport (3). Atherosclerosis might

be the most relevant pathophysiological process in which SRBI is crucial, and therefore is also the most investigated one. Hence in this chapter I will focus on atherosclerosis, but also on female fertility, as in 10-20% of human infertile females the cause is unknown and abnormal lipoprotein metabolism due to mutations in SRBI might be the underlying problem (3,4).

Atherosclerosis:

Atherosclerosis is a systemic disease of the arteries due to a chronic inflammatory response in the vessel walls. This inflammation process is initiated by invasion of the endothelium by oxidized LDL, thereby damaging the arterial wall. In response to this damage, macrophages accumulate and absorb oxidized LDL particles. These macrophages then develop into so-called foam cells, which attract further macrophages, thus prolongating the inflammatory response, resulting in tissue remodelling, atherosclerotic plaque formation and blood clotting that narrows the blood vessels (3). This is a process that takes decades, but finally can lead to ischemia, thrombosis, embolism, coronary artery disease, and appoplexy.

Coronary artery disease is the major cause of death in western industrialized countries and it is long known that low plasma HDL levels and coronary artery disease are associated. The mechanism by which HDL mediates its cardioprotective effect is not yet known, but it is most probably due to reverse cholesterol transport (3). Therefore, SRBI might influence atherosclerosis and all associated diseases by mediating uptake of cholesteryl esters from peripheral tissues by HDL, transport and transfer of this CEs to the liver, and secretion into bile. Some experiments were performed to gain insight into the role of SRBI in prevention or development of atherosclerosis. Different mouse models have been used which had a genetic background that made them susceptible to atherosclerosis. The following mouse models exhibit increased levels of apoB-containing particles with atherogenic properties and therefore have increased levels of plasma total cholesterol (3):

1. The LDLR knock out mouse
2. The human apoB transgenic mouse fed a high fat/high cholesterol (Western type) diet
3. The apoE knock out mouse

ApoE/SRBI double-knock out mice fed a normal chow diet, compared to apoE single knock out mice show a dramatic acceleration of atherosclerosis, especially in the aortic sinus and coronary arteries, already at week 4-7 after birth. This model is quite a good one for human atherosclerosis, as the coronary arterial lesions of these mice resemble remarkably those in human patients (3).

The cardioprotective effect of SRBI was shown by an experiment using LDLR knock out mice. Transient hepatic overexpression of SRBI, using an adenoviral system, in these mice fed a Western type diet, decreased plasma HDL cholesterol significantly. Surprisingly, even though non-HDL particles were not affected, areas of atherosclerotic lesions were reduced relative to untreated LDLR knock out control mice. Lesion size of individual mice correlated with their mean HDL-cholesterol level, but in some cases also with their mean non-HDL cholesterol level. The anti-atherosclerotic effect mediated by SRBI thus seems to be based on changes in net cholesterol flux, or maybe also by reduction of non-HDL cholesterol levels (3).

In another experiment, human apoB transgenic mice were used to study the effects of varying levels of stable SRBI expression in the liver. Both low and high levels of SRBI expression were failed to produce any protective effect. Only moderate levels of SRBI expression in the liver showed anti-atherogenic effects (3). Therefore, the role of SRBI in atherogenesis is very complex and may depend not only on the levels of SRBI expression, but also on the atherogenesis model used.

Female Fertility:

Female, but not male, SRBI-KO mice are infertile, even though they exhibit normal estrus cycle, ovulation, and progesterone levels during pseudopregnancy. This infertility is due, at least in part, to the ovulation of dysfunctional oocytes (4). Transplantation of ovaries from SRBI knock out mice into ovariectomized SRBI-positive mice rescued the ovarian wild type phenotype, and 6 out of 7 mice became pregnant. The litters were all heterozygous for the SRBI mutation, proving that the oocytes from which these mice developed, originated from the SRBI-negative ovaries transplanted into the SRBI positive mice. Thus, ovarian

SRBI expression doesn't seem to be essential for embryonic development, adult maturation or ovarian function, if an extraovarian environment is provided which exhibits SRBI expression. In addition, treatment of SRBI knockout mice with probucol, which lowers HDL-cholesterol levels, restored fertility (4).

Based on these experiments, it seems as if abnormal lipoprotein metabolism in SRBI knock out mice is responsible for female infertility. As already mentioned above, absence of SRBI in mice results in large and heterogeneously-sized HDL particles abnormally enriched in apoE, and plasma total cholesterol levels are elevated 2-2.5 times. So, maybe these HDL particles are not able to transport important fertility factors to the oocytes, or these abnormal lipoproteins actively somehow inhibit critical ovarian functions (1,3,4).

In any case, additional investigations of potential relationships between abnormal lipoprotein metabolism and human female infertility of unknown etiology may be informative and may possibly lead to new therapeutic approaches (4).

1.7 Scavenger Receptor Class B Type II

SRBI and SRBII are alternatively spliced products from a single gene (Fig. 1.7) and differ only in their carboxy-terminal cytoplasmic domain. Exon 12 of SRBI, a 129 nt long sequence of the SRB gene transcript, is removed and thereby the stop codon of SRBI is lost. A 3'-untranslated region of SRBI then gets attached to exon 11 and thereby becomes a coding exon (exon 13), with another stop codon at its 3' end. This transcript then becomes translated into the protein SRBII (2). Human SRBI, as mentioned above, is 509 amino acids long, and the last 47 amino acids, all of them part of the cytoplasmic domain of SRBI, get replaced by 44 amino acids of the alternative exon 13 of SRBII (2).

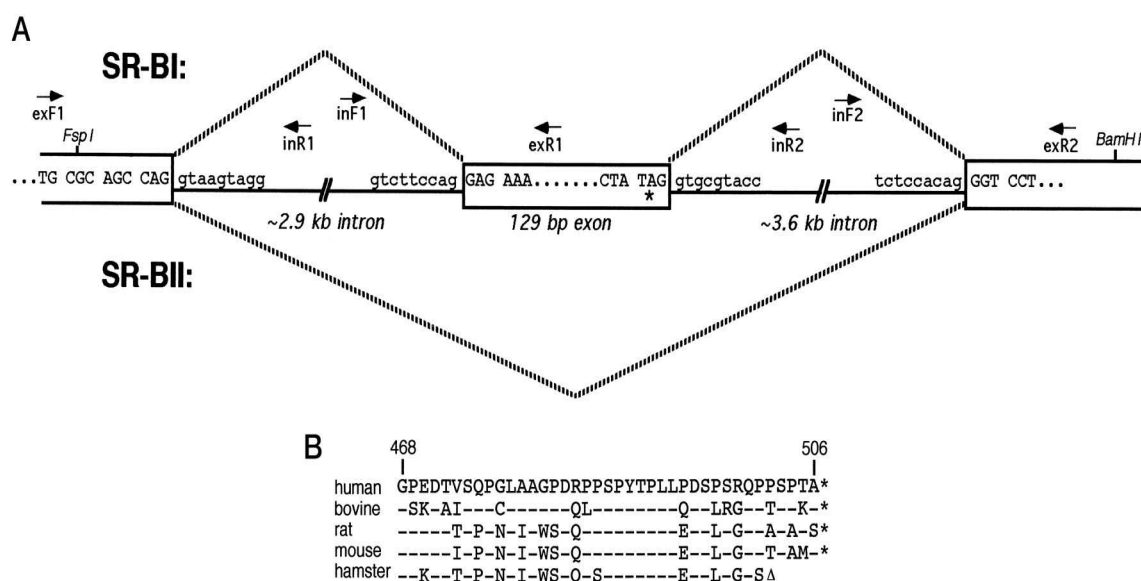


Fig.1.7 Alternative Splicing of SRBI and SRBII

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The isoform SRBII has been found in many organisms, like human, cattle, rat, and mouse. The open reading frame coding for SRBII in these organisms is of the same length, encoding 506 amino acids; furthermore, the nucleotide sequences in this SRBII-specific exon are highly conserved (2).

Just like SRBI, SRBII is found in the plasma membrane and clusters in caveolae-like cholesterol-rich domains. SRBII also gets extensively N-glycosylated and palmytoylated, and both isoforms have similar binding affinities for HDL and mediate selective lipid uptake, but the efficiency of SRBII in mediating both cholesterol influx from HDL to cells and cholesterol efflux from cells to HDL is approximately 4 times lower than that of SRBI (2).

SRBII protein accounts for 5-12% of total SRB protein in mouse liver, testis and adrenal glands, but 30-80% of total SRB mRNA. Half-lives of the mRNAs of the two isoforms are similar. Thus, the difference in protein levels might be resulting from different translation efficiencies of SRBI and SRBII mRNAs. Anyhow, little is known about splice site selection and mRNA:protein ratio differences, but estrogen seems to play a key role (2).

Estrogen-treatment of rats, especially in the more potent synthetic form of 17- α -ethynyl estradiol, can influence SRBI and SRBII levels in a dose- and

glycosylation- state dependent manner, in the liver. Both glycosylated and nonglycosylated SRBI levels decrease, non-glycosylated SRBII increases and glycosylated SRBII doesn't seem to be influenced. Glycosylation is proposed to play a role in the folding and export of the SRBI protein from the endoplasmatic reticulum, and it is not yet known whether or not glycosylation of SRBII is important for its stability, subcellular localization or lipid transfer activities, but it appears that post-translational modification of SRBII may be one of the factors in estrogen's effect on rat liver cells (5).

Splicing factors involved in alternative splicing of SRB are ASF/SF2, Tra-2 α and Tra-2 β . Overexpression of these splicing factors in rat liver results, in the case of ASF/SF2, in upregulation of SRBI expression and downregulation of SRBII expression, but in the case of Tra-2 α and Tra-2 β in a downregulation of SRBI expression and upregulation of SRBII expression. 17- α -ethynyl estradiol treatment of rats

induced an increase in Tra-2 β expression in the liver, but did not influence ASF/SF2 levels of expression. The result is obtained when HepG2 cells are stimulated with 17- α -ethynyl estradiol. Therefore it seems that estrogen affects SRB splicing at least partly through the regulation of Tra-2 β (5).

The findings listed above suggest that estrogen is able to influence alternative splicing of SRB mRNA and post-translational modification of SRB proteins.

2 Materials and Methods

2.1 Animals

Derco brown laying hens (30-40 weeks old) were purchased from Heindl Co. (Vienna, Austria) and maintained on layer's mash with free access to water and feed under a daily light period of 16 hours.

2.2 Molecular Biology:

2.2.1 Bacterial Strains and Vector Systems

Strain	One Shot TOP10 Chemically Competent E.coli	One Shot BL21 Competent Cells
Genotype	<i>F-mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi80/lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(araleu)7697galU$ <i>galK</i> <i>rpsL</i> (<i>Str^R</i>) <i>endA1</i> <i>nupG</i>	<i>F- ompT hsdSB (rB-mB-) gal dcm (DE3)</i>
Reference/Source	Invitrogen	Invitrogen

Table 2.2.1 Bacterial strains used for transformation

Vector	PET-25b(+)	pCR2.1-TOPO
Size	5547 basepairs	3931 basepairs
Genotype characteristics	T7 promotor, T7 transcription start, <i>peI</i> B coding sequence, multiple cloning sites, HSV tag, His tag, T7 terminator, <i>lacI</i> coding sequence, pBR322 origin, <i>bla</i> coding sequence, f1 origin	<i>LacZ</i> α gene, M13 reverse priming site, MCS, T7 promoter/priming site, M13 forward priming site, f1 origin, kanamycin resistance, ampicillin resistance, pUC origin
Reference/Source	Novagen	Invitrogen

Table 2.2.2 Vector systems used for cloning

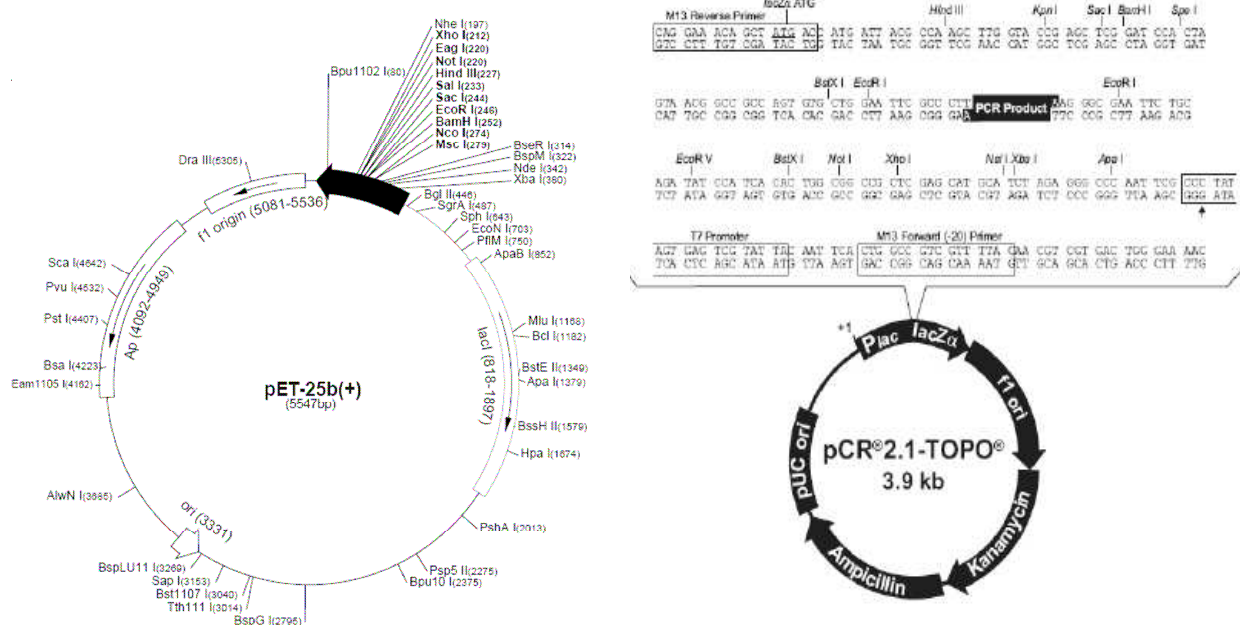


Figure 2.2.1 Genetic map of pET-25b(+) and pCR2.1-TOPO Vector

2.2.2 Oligonucleotide Primers

Name	Sequence	T _m [°C]	%GC
ggSRB1-FWD1	5'-TGA GGA TTG ACC CCA GCA-3'	56	
ggSRB1-REV1	5'-GCA TCG GGG CTG TAG AAC-3'	58,2	
ggSRBI-Fwd1-NcoI	5'-TAC CAT GGA TGT GAG GAT TGA CCC CAG C-3'	68	53,6
ggSRBI-Rev1-EcoRI	5'-ATG AAT TCT TGG CAT CGG GGC TGT AGA AC-3'	66,7	48,3
ggSRBI-Fwd4	5'-CGC CAT CGT CAA GGA GCA-3'	58,2	61,1
ggSRBI-Rev4	5'-GGG GTC GTG TGG GTG CTT-3'	60,5	66,7
ggSRBI-Rev5	5'-GAC ACG TCC CCT TCT GGA G-3'	61	63,2
ggSRB-F1	5'-GCG CTC TGC GAG CCA TGG-3'	62	
GgSRBII-Fwd1	5'-GAT GGC TCA GTC CTA TCG-3'	56	55,6
GgSRBII-Rev1	5'-CAG AAG TGG GGT GTA G-3'	52	56,3
GgSRBII-Rev2	5'-TGT CCG CTG AGA GAC TCC-3'	58,2	61,1
GgSRBII-Rev3	5'-CAT CAG GGA CTC AGA GTA GGC-3'	61,8	57,1
GgSRBII-Fwd3	5'-TGT TCA TCT CCC ACC CTC AC-3'	59,4	55
ggSRBII-Fwd1.2	5'-GAT GGC TCA GTC CTA TCG CAG-3'	61,8	57,1

Table 2.3.1 Oligonucleotides used for PCR of laying hen liver tissue cDNA. The designed oligonucleotides were synthesized by MWG-Biotech AG, Ebersberg, Germany.

Oligonucleotide primers were designed using a Gallus gallus SRBI sequence derived from ENSEMBL (ENSGALG00000003018) and the online programs ClustalW and Pearl Primer.

2.2.3 RNA Isolation

Total RNA was isolated using NucleoSpin RNA II kit from Macherey-Nagel or TRI Reagent from Molecular Research Center, Inc.

2.2.4 RNA Isolation using TRI Reagent from Molecular Research Center, Inc.

200mg of frozen chicken tissue were placed in a 15ml Falcon tube, chilled on ice and homogenized in 2ml TRI reagent (1ml per 100mg tissue), using an Ultra-Turrax-T25-Homogenizer. In between two different tissues the ultra-turrax was cleaned with diethyl pyrocarbonate (DEPC) [0,1%] water and 70% ethanol). After homogenization the lysates were incubated for 5 minutes (min) at room temperature. 400µl chloroform were added and the Falcon tubes were vigorously shaken, followed by 15min of incubation at room temperature. After incubation, the tubes were centrifuged for another 15min at 12000xg and 4°C. The aqueous phase was transferred into a new Falcon tube and 1ml isopropanol was added. The tube was vigorously shaken and another time incubated for 10min at room temperature. After incubation the tubes were centrifuged for 30min at 12000xg and 4°C. The supernatant was discarded and the pellet washed in 2ml pure ethanol. After 5min of centrifugation at 12000xg and 4°C ethanol was removed and the RNA pellet dried at the air. Finally the RNA pellet was dissolved in 50µl sterile DEPC (0,1%) water, put into a sterile Eppendorf tube and incubated at 60°C for 10min, gently shaking. After photometric determination of the RNA concentration the samples were kept at –80°C.

DEPC H₂O dd

2ml diethyl pyrocarbonate [100%] (DEPC)
double distilled (dd) H₂O up to 2000ml

DEPC.H₂O was incubated at room temperature overnight and autoclaved the next day. DEPC.H₂O was stored at room temperature.

Determination of RNA concentration

For determination of RNA concentration samples were diluted 1:1000 or 1:100 (depending on the RNA concentration in the samples) with DEPC.ddH₂O. Absorbance was measured in quartz cuvettes at a wavelength of 260nm. The RNA concentration was then calculated as following:

Extinction at 260nm × dilution factor (100 or 1000) × 40 = ng / µl RNA

2.2.5 RNA Isolation using NucleoSpin™ RNA II kit from Macherey-Nagel

30mg frozen chicken tissue were placed in a sterile glass potter. 350µl buffer RA1 and 3.5µl β-mercapthoethanol were added and the tissue was homogenized. The lysate was then loaded onto a nucleospin filter unit and centrifuged for 1min at 11000xg. The filter unit was discarded and 350µl 70% ethanol were added to the lysate, which was then shaken vigorously. The lysate was then loaded onto a nucleospin RNA II column and centrifuged for 30seconds (sec) at 8000xg. 350µl membrane desalting buffer were added and the column was centrifuged for 1min at 11000xg. The flowthrough was discarded and the membrane was incubated for 15min at room temperature with 95µl of DNase reaction mix (10µl DNase I and 90µl DNase reaction buffer). After incubation, 200µl buffer RA2 were added and the solution was centrifuged for 30sec at 8000xg. Flowthrough was discarded. 650µl buffer RA3 were added, followed by another centrifugation step of 30sec at 8000xg. Then, 250µl of buffer RA3 were added and the column was centrifuged for 2min at 11000xg, and the nucleospin column was placed on a nuclease free collection tube. To elute bound RNA, 60µl RNase free water were added and collected through centrifugation for 1min at 11000g.

2.2.6 cDNA Synthesis

cDNA synthesis was carried out using SUPERScript RNase H- Reverse Transcriptase from Invitrogen. Total RNA was isolated out of 30mg chicken liver tissue using a Nucleo Spin RNA II Kit from Macherey-Nagel and the following components were mixed in a sterile Eppendorf tube:

10µl total RNA

1µl dNTPs (10mM each)

1µl Oligo (dT) (500µg/ml)

The mixture was heated to 65°C for 5 minutes and then chilled on ice. The following components were added:

4µl First Strand Buffer

2µl 0.1M DTT

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After an incubation at 42°C for 2min, 1 µl SUPERSCRIPT II reverse transcriptase (200 U/µl) was added and mixed by pipetting. The cDNA synthesis reaction was performed at 42°C for 50 minutes and finally stopped by heating to 70°C for 15 min. For elimination of RNA, 2 units of RNase H (Roche) were added and incubated at 37°C for 20 minutes. cDNA samples were stored at -20°C.

2.2.7 Polymerase Chain Reaction (PCR)

Different PCR reaction mixtures and programs were used, depending on the different primer sets. 25µl reactions were used. The following components were pipetted on ice and gently mixed in a sterile PCR reaction tube:

0.5µl cDNA
2.5µl 10xPCR Reaction Buffer [15mM MgCl₂]
0.5µl ggSRB1-FWD1 [25pM]
0.5µl ggSRB1-REV1 [25pM]
1µl dNTP mix [10mM each dNTP]
0.5µl Polymerase [1U/µl]
0/0.5/1/2µl DMSO
x µl sterile ddH₂O to a final volume of 25µl

Finnzymes Dynazyme Taq Polymerase EXT was used for optimization of polymerase chain reactions, whereas Fermentas High Fidelity Taq Polymerase was used if the PCR product was to be sequenced.

The following PCR programs were used:

Initial Denaturation	94°C	5'	1x
Denaturation	94°C	30''	30x
Annealing	56°C	30''	
Extension	72°C	1'	
Final Extension	72°C	10'	1x
Pause	4°C		

Table 2.2.7.1 PCR program used for primer set ggSRBI- Fwd1/Rev1.

Initial Denatura- tion	94°C	5′	1x
Denaturation	94°C	30″	40x
Annealing	63°C	30″	
Extension	72°C	1′	
Final Extension	72°C	10′	1x
Pause	4°C		

Table 2.2.7.2 PCR program used for primer set ggSRBI-f1-NcoI/r1-EcoRI

Initial Denatura- tion	94°C	5′	1x
Denaturation	94°C	1′	40x
Annealing	56°C	30″	
Extension	72°C	2′	
Final Extension	72°C	10′	1x
Pause	4°C		

Table 2.2.7.3 PCR program used for primer set ggSRBI-Fwd4/Rev4

Initial Denatura- tion	94°C	5′	1x
Denaturation	94°C	1′	3x
First Annealing	64°C	30″	
Elongation	72°C	2′30″	
Denaturation	94°C	1′	3x
Second Anneal- ing	61°C	30″	
Elongation	72°C	2′30″	
Denaturation	94°C	1′	34x
Final Annealing	58°C	30″	
Elongation	72°C	2′30″	
Final Elongation	72°C	10′	1x
Pause	4°C		

Table 2.2.7.4 PCR program used for primer set ggSRBI-F1/Rev5

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Initial Denaturation	94°C	5′	1x
Denaturation	94°C	30″	40x
Annealing	51°C	30″	
Extension	72°C	30″	
Final Extension	72°C	10′	1x
Pause	4°C		

Table 2.2.7.5 PCR program 1 used for primer set ggSRBII-Fwd1/Rev1

Initial Denaturation	94°C	5′	1x
Denaturation	94°C	30″	6x
First Annealing	49°C	30″	
Elongation	72°C	30″	
Denaturation	94°C	30″	34x
Final Annealing	52°C	30″	
Elongation	72°C	30″	
Final Elongation	72°C	10′	1x
Pause	4°C		

Table 2.2.7.6 PCR program 2 used for primer set ggSRBII-Fwd1/Rev1

Initial Denaturation	94°C	5′	1x
Denaturation	94°C	30″	3x
First Annealing	52°C	30″	
Elongation	72°C	1′	
Denaturation	94°C	30″	3x
Second Annealing	55°C	30″	
Elongation	72°C	1′	
Denaturation	94°C	30″	34x
Final Annealing	58°C	30″	
Elongation	72°C	1′	
Final Elongation	72°C	10′	1x
Pause	4°C		

Table 2.2.7.7 PCP program used for primer set ggSRBII- Fwd3/Rev3

Table 2.2.7.8 PCR program used for primer set ggSRBII-Fwd1.2/Rev3

Initial Denaturation	94°C	5'	1x
Denaturation	94°C	1'	3x
First Annealing	55°C	30''	
Elongation	72°C	30''	
Denaturation	94°C	1'	3x
Second Annealing	58°C	30''	
Elongation	72°C	30''	
Denaturation	94°C	1'	34x
Final Annealing	61°C	30''	
Elongation	72°C	30''	
Final Elongation	72°C	10'	1x
Pause	4°C		

All PCR products were analyzed by agarose gel electrophoresis.

2.2.8 Agarose Gel Electrophoresis

Separation of DNA fragments was performed by gel electrophoresis using 1% (w/v) agarose gels with a constant voltage of 130V. DNA samples were mixed with 5x DNA loading buffer containing Bromophenol Blue or Xylene Cyanol FF serving as front marker. 1x TAE was used as electrophoresis running buffer. The 1kb Plus DNA Ladder from Invitrogen was used as size marker. To visualize DNA fragments under ultraviolet light (366 nm), the agarose gel contained (1:10 000)the fluorescent intercalating dye ethidium bromide (40µg/100 ml).

50x TAE, pH 8.0

2M Tris-HCl

1M Acetic acid

0.1M EDTA

Ethidium bromide stock solution

10mg/ml ethidium bromide in H₂Odd.

5× DNA loading buffer

5ml 100% Glycerol

2ml 0.5M EDTA

12ml 50× TAE

3ml H₂O

Bromophenol blue or Xylene Cyanol FF

2.2.9 DNA Gel extraction

All gel extractions were performed using QIAquick Gel Extraction Kit (QIAGEN). All centrifugation steps were carried out in a table-top microcentrifuge. The desired DNA band was excised from the agarose gel with a sterile scalpel and transferred to an Eppendorf tube. 3 volumes of buffer QG were added to 1 volume of gel (100 mg gel weight = 1 volume of gel = 300 µl buffer QG). The gel was dissolved completely by incubating at 50°C for 10 minutes and repeated vortexing every 2-3 minutes during incubation. 100 µl Isopropanol were added and the sample was mixed. The solution was then loaded onto a QIAquick column and centrifuged for 1 min at 14000 rpm. The flowthrough was discarded, 750 µl buffer PE(+EtOH) were added and the column was centrifuged again for 1 min at 14000 rpm. The flowthrough was discarded and to completely dry the column, another centrifugation step was done for 1 min at 10000xg. The DNA was then eluted by loading 30 µl EB buffer (10mM Tris-HCl pH 8.5), incubation for 1 min at room temperature and centrifugation for 1 min at 14000 rpm.

2.2.10 Cloning

2.2.10.1 Topo TA Cloning

Taq-polymerase used for PCR has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosin (A) to the 3' ends of PCR products. Linearization of pCR2.1-TOPO vector results in a single overhanging 3' deoxythymidine (T) residue. Topoisomerase I, which is covalently bound to this vector allows Taq-polymerase derived PCR products to insert into the linearized

vector without adding DNA ligase to the reaction. The following components were pipetted into Eppendorf reaction tubes:

4 μ l PCR product (purified by gel extraction)
1 μ l salt solution (1.2 M NaCl; 0.06 M MgCl₂)
1 μ l TOPO vector (pCR2.1-Topo)

This reaction mixture was incubated for 10 minutes at room temperature and then used for transformation.

2. 2.10.2 Cloning using pET-25b(+) as vector

To generate an antibody specific for SRBI and SRBII, pET 25b(+) was chosen as vector system for recombinant protein expression. Due to the vector characteristics the recombinant protein will be secreted into the periplasma of the transformed bacteria and contain a 6xHistidine tag attached to its carboxy-terminal end, which should ease purification of the expressed protein. The following components were pipetted into Eppendorf reaction tubes. Each enzyme and buffer was derived from Roche.

0.5 μ g pET-25b(+) DNA (Midiprep)
1 μ l each NcoI/EcoRI restriction enzyme
2 μ l SURE Cut Buffer H
x μ l ultrapure ddH₂O to a final volume of 20 μ l

3 μ g Insert DNA (Midiprep)
1 μ l each NcoI/EcoRI restriction enzyme
2 μ l SURE Cut Buffer H
x μ l ultrapure ddH₂O to a final volume of 20 μ l

These mixtures were gently shaken for 24 hours at 37°C. Insert excision and vector linearization, caused by the restriction enzyme treatment was then checked using 1%

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agarose gel electrophoresis. 20µl of the insert restriction enzyme mixture and 1µl of the vector restriction enzyme incubation mixture were loaded onto that gel. The excised insert of correct length was then extracted using QIAquick Gel Extraction Kit and stored at -20°C. The remaining 19µl of vector restriction enzyme reaction were then dephosphorylated. The following components were pipetted into an Eppendorf reaction tube.

19µl vector restriction enzyme mixture
4µl SAP buffer
3µl SAP (shrimp alkaline phosphatase)
xµl ultrapure ddH₂O to a final volume of 40µl

This dephosphorylation mixture was then incubated gently shaking for 15min at 37°C. Afterwards SAP was inactivated by gently shaking the mixture for 20min, at 65°C. The vector then was precipitated and therefore the following components were pipetted into an Eppendorf reaction tube:

40µl dephosphorylation mixture
54µl isopropanol
28µl 1M autoclaved sodium-acetate
14µl ultrapure ddH₂O

The reaction tube was then vortexed and stored at -20°C overnight. The next day it was centrifuged for 30min at 13000rpm and 4°C. The supernatant was discarded and the pellet was washed with 1ml 70% ethanol, followed by another centrifugation step for 10min at 13000rpm and 4°C. Again the supernatant was discarded and the pellet was dried for 10min at 37°C. Now the insert and vector DNA could be ligated. Thus the following components were pipetted onto the dry vector pellet:

17µl Insert
2µl 10xligation buffer
1µl T4 ligase

This mixture then was placed into a 14°C water bath and kept there overnight. 10µl of this mixture were used for transformation of chemically competent E.coli BL-21.

2.2.11 Transformation of chemically competent E.coli

25µl bacterial solution (kept frozen at –80°C) were slowly thawed on ice, added to the cloning mixture and mixed gently. The reaction was incubated on ice for 30min. Then, the cells were heated for 30sec at 42°C and chilled on ice for 2 min. For regeneration, 200µl of provided S.O.C. medium were added and the cells were incubated for 1 hour at 37°C under gentle shaking. 50µl and 150µl of the transformation mixture were plated onto LB-Amp/X-Gal plates (50µl of X-Gal [50mg/ml] per LB-Amp plates) and incubated overnight at 37°C.

LB-Amp plates

10g Peptone from Casein

5g Yeast Extract

10g NaCl

12g Agar-Agar

H₂O dd ad 1000ml

100µg/ml Ampicillin

2.2.12 Preparation of Plasmid DNA

Preparation of plasmid DNA was carried out using FastPlasmid Mini Kit from Eppendorf and QIAfilter Plasmid Midi Kit from QIAGEN. DNA concentration was determined analogous to RNA concentration determination by UV spectrophotometry at 260nm and calculation of the concentration according to the following equation:

Extinction at 260nm × dilution factor × 50 = ng / µl DNA

LB medium

10g Trypton

5g Yeast Extract

10g NaCl

H₂O dd. ad 1000 ml

LB-Amp

LB medium

100µg/ml Amp

TE buffer

10mM Tri-HCL, pH 8

1mM EDTA

2. 2.12.1 Miniprep

A single white bacterial colony from an LB-Amp plate was transferred into 5ml of LB-Amp medium and incubated at 37°C overnight, shaking vigorously. 1.5ml of the bacterial culture were transferred into an 2ml Eppendorf tube and harvested by centrifugation for 1 minute at 12000xg. The supernatant was removed and the pellet was resuspended in 400µl of ice-cold complete lysis solution by vortexing at highest setting for 30sec. The cell lysate was incubated at room temperature for 3 min, transferred to a Spin Column Assembly by pipetting and centrifuged for 1min at 14000rpm. The flowthrough was discarded and bound DNA was washed by adding 400µl of Wash buffer and centrifugation for 1min at 14000rpm. To dry the column, the flowthrough was again discarded and the column one more time centrifuged for 1min at 14000rpm. The column was put onto a collection tube and DNA was eluted by adding 50µl of EB buffer and a final centrifugation for 1min at max. speed. Plasmid DNA samples were stored at -20°C.

2. 2.12.2 Midiprep

A single white bacterial colony from an LB-Amp plate was transferred into 2ml LB-amp medium and was incubated for 8 hours, at 37°C, vigorously shaking. Then, 25ml of LB-amp medium were inoculated with 50µl of that starter culture. This culture was then incubated for 16 hours at 37°C, again vigorously shaking. Bacterial cells were harvested by centrifugation at 6000xg for 15min at 4°C. Bacterial pellets were resuspended in 4ml buffer P1. Another 4ml buffer P2 were added and the solution was mixed vigorously by inverting the sealed tube 6 times, followed by another incubation step for 15min at room temperature. Subsequently, 4ml of chilled buffer P3 were added to the lysate and again the lysate was mixed by inverting the sealed tube 6 times. The lysate was then poured into the barrel of a QIAfilter Cartridge and allowed to incubate for another 10min at room temperature. Then, the cell lysate was gently filtered into a previously equilibrated QIAGEN-tip. The now cleared lysate then entered the resin by gravity, followed by 2 times washing of the QIAGEN-tip with buffer QC (10ml each wash step). 5ml of buffer QF were added to elute DNA into a 15ml Falcon tube. 3.5ml isopropanol were applied for precipitation of DNA, followed by immediate mixing and centrifugation at 15000xg for 30min at 4°C. The

supernatant was discarded carefully and the pellet was washed with 2ml 70% ethanol, followed by another centrifugation at 15000xg for 10min at room temperature. Again the supernatant got discarded and the pellet was allowed to air dry for 10min. Finally the air dried pellet got resuspended in 100ml of TE buffer, pH 8.0.

2.2.13 Control Restriction Enzyme Digestion

For digestion of minipreps the following components were mixed and incubated for 60 minutes at 37°C, gently shaking:

3µl plasmid DNA

1µl buffer

0,5µl restriction enzyme

5,5µl ddH₂O

The appropriate restriction buffer was chosen in agreement with the recommendations of the producer. The restriction digest pattern was checked by agarose gel electrophoresis.

2.2.14 Sequence Analysis

For sequence analysis, Mini or Midi - preparations of plasmid DNA were sent to VBC - Genomics Bioscience Research GmbH, Vienna, Austria.

2.2.15. Radiolabelling of DNA Fragments

To generate a radiolabelled ggSRBI probe for Northern blot analysis of total RNA, the Amersham Megaprime DNA labelling kit was used. 4µl purified PCR fragment diluted with 24µl DEPC treated H₂O and 5µl primer (random hexamers provided by the kit) were combined in an Eppendorf screwcap tube. The mixture was heated to 95°C for 5 minutes and then chilled on ice. The content was collected by short centrifugation. 10µl labelling buffer, 2µl Klenow Polymerase (5U/µl) and finally 5µl α-³²P dCTP (10µCi/µl; Hartmann Analytics) were added. The mixture was spun and incubated at 37°C for 1 hour. The reaction was stopped by heating to 95°C for 5 minutes. The

labelled DNA was separated from unincorporated radioactive nucleotides by gel filtration using NICK columns (Pharmacia Biotech). The sample was applied to a Pharmacia NICK column, which was rinsed once with TE buffer. The labelled DNA was eluted in five 400 μ l fractions. Fractions were measured with a radiometer. According to the radio labelling protocol, radiation should be highest in fraction 2, if α -³²P dCTPs have been incorporated into the probe DNA strands successfully. This fraction was then added to the prehybridized Northern blot membrane (see 2.15)

2.2.16 Glyoxal Northern Blot Analysis

Northern Blotting was carried out using the glyoxal gel electrophoresis method. All solutions were prepared with RNA grade chemicals. ddH₂O was treated with DEPC (DEPC:ddH₂O, 0.1%). For sample preparation the following components were pipetted into a fresh autoclaved Eppendorf reaction tube, chilled on ice:

Between 10 to 30 μ g total RNA in 6 μ l ddH₂O ultrapure

11.8 μ l DMSO

2.4 μ l 0.1 M NaHPO₄ buffer

3.5 μ l 40% Glyoxal

Samples were incubated at 50°C for 1 hour and subsequently chilled on ice. Finally, 6.3 μ l glyoxal loading buffer were added. Marker preparation was done in analogy to sample preparation except the incubation step at 50°C, which was 30min of length and 3 μ l of 0.5-10Kb RNA Ladder from Invitrogen were added instead of 10 to 30 μ g total RNA. Then, samples and marker were loaded onto the agarose gel.

A 1.2% agarose gel, 10mM NaHPO₄ buffer (which was also used as running buffer) was prepared. Samples and marker were loaded into the slots and the gel was run at 80V for 90 minutes. The direction of the gel was changed every 30 minutes to avoid pH- or salt-gradients.

After gel electrophoresis, the gel was equilibrated in Gel Wash Buffer-I for 20 minutes, in Gel Wash Buffer-II for 20 minutes and in 10 \times SSC for 30 minutes at room temperature, gently shaking. To transfer the RNA onto a nylon membrane a blot was prepared as follows:

A glassplate was covered with 6 Whatman papers of same size as the plate soaked in 20× SSC, the gel was applied upside down, followed by a nylon membrane (Hybond-N for nucleic acid transfer from Amersham Biosciences) and 6 dry Whatman papers of the size of the membrane. A package of green towels and a weight were placed on top of the blot, so capillary transfer could occur sufficiently. Blotting was carried out overnight at RT. After marking the slots of the loaded samples, the next day, the membrane was rinsed in 2× SSC, placed on a Whatman paper and crosslinked in a Stratagene UV crosslinker.

Now RNA was stained with methyleneblue for 5 minutes and destained with DEPC treated H₂O_{dd}. The stained membrane was photocopied and the replica served as size marker for 28S and 18S rRNA, representing a loading control.

Then the membrane was prehybridized to block free positions on the membrane, in a glass roller-bottle filled with 10ml of prewarmed hybridization solution for 3 hours at 65°C. After addition of the radiolabelled probe (see 2.14), the blot was hybridized overnight at 65°C circulating.

The next day the membrane was washed 1-2 times for 10 minutes with wash buffer A and 2-3 times for 10 minutes with wash buffer B, depending on the radiation intensity of the blot background. The washing solutions were prewarmed to 65°C.

Finally the membrane was fixed in a film cassette and exposed to a film (Pierce, CL-XPosureTMFilm) at –80 °C for one to several days depending on the intensity of the signal.

1M Na-Phosphate Buffer

462mM Na₂HPO₄

469mM NaH₂PO₄

pH 6.8

add DEPC treated H₂O_{dd} up to 200ml

Glyoxal Loading Buffer

50% Glycerol

10mM Na-Phosphate Buffer

0.4% Bromophenolblue

0.25% Xylene Cyanol FF

Methylenblue Staining Reagent

0.04% Methylenblue

0.5M NaOAc pH 5.2

Gel Wash Buffer-I

50mM NaOH

150mM NaCl

DEPC treated H₂O_{dd} to a final volume of 250ml

Materials and Methods

20× SSC

3M NaCl
0.3M Na-Citrat
pH 7,2
add DEPC treated H₂O up to 250ml

Gel Wash Buffer-II

100mM Tris-HCl buffer pH 7.5
150mM NaCl
add DEPC treated H₂O up to 150ml

Hybridization Solution

1% BSA Fraction V
40% DEPC treated H₂O
7% SDS
500mM Na-Phosphate Buffer pH 6.8
1mM EDTA
mixed in order shown above; final volume
should be 10ml

Membrane Wash Buffer A

0.5% BSA
5% SDS
40mM Na-Phosphate buffer
1mM EDTA
add DEPC treated H₂O up to 250ml

Membrane Wash Buffer B

1% SDS
40mM Na-Phosphate buffer
1mM EDTA
add DEPC treated H₂O up to 500ml

2.3 Protein Chemistry

2.3.1 Protein Extraction

2.3.1.1 Total Protein Extraction

8ml ice-cold homogenization buffer and 100µl protease inhibitor cocktail were added to 2g freshly obtained chicken tissue, which was then homogenized with an Ultra-Turrax T25 homogenizer 3× for 20sec. In between the homogenization steps the samples were chilled on ice. The homogenates were centrifuged for 3min at 3000×g and 4°C. The supernatant was transferred to a fresh tube, 400µl 20% Triton X-100 were added and the solution was vigorously shaken. The mixture was then incubated at 4°C for 30 minutes and finally centrifuged at 300 000×g for 1 hour at

4°C. The supernatant was aliquoted and stored at –8 0°C. Protein concentration was determined using Bio-Rad Protein Assay (BioRad). Protease inhibitor cocktail was added to homogenization buffer, so proteins were not degraded.

Homogenization Buffer

20mM HEPES

300mM sucrose

150mM sodium-chloride

Protease Inhibitor Cocktail

AEBSF 104mM

0.08mM Aprotinin

2mM Leupeptin

4mM Bestatin

1.5mM Pepstatin

1.4mM E-64

1ml/20g wet weight

2.3.1.2 Membrane Protein extraction

Homogenization of chicken tissue for membrane preparation was performed analogous to total protein extraction (see 2.3.1.1). After homogenization total homogenates were centrifuged at 620xg for 10min at 4°C. The supernatant was then transferred to a reaction tube, and 1/20 of total volume 20% Triton X-100 was added. The homogenates were vortexed and incubated for 30min at 4°C. After incubation samples were centrifuged at 300 000xg for 60min at 4°C. The resulting supernatant was aliquoted and stored at –80°C. Protein concentration was determined using Bio-Rad Protein Assay (BioRad). Protease inhibitor cocktail was added to homogenization buffer, so proteins were not degraded.

Homogenization Buffer

20mM HEPES

300mM sucrose

150mM sodium-chloride

0.5mM PMSF

2.5µM leupeptin

2.3.2 Protein Analysis**2.3.2.1 SDS-PAGE**

Protein extracts, suspensions of bacteria expressing recombinant peptides, ,and protein purification or wash fractions were analyzed by sodium dodecylsulfate

Materials and Methods

polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie staining or transfer of the separated proteins to a nitrocellulose membrane Western blotting of proteins of interest. Protein samples were mixed with Laemmli buffer (reducing or non-reducing). Under reducing conditions the samples were incubated for 5min at 95°C, chilled on ice immediately, spun down in a table top centrifuge and loaded onto a sodium dodecylsulfate polyacrylamide gel. Under non-reducing conditions samples mixed with Laemmli buffer were loaded as such. Stacking gels had a concentration of 4% polyacrylamid, whereas separation gels of 10-, 12- and 14% were used. 45 to 50µg of total and membrane protein extracts, as well as up to 20µl of recombinant bacterial suspensions, protein purification or wash fractions were loaded per slot. The gel was run at 80V until the bromophenolblue front reached the separation gel and then was turned to 100 -140V. 5µl Fermentas Page Ruler Prestained Protein Ladder Plus were loaded as Marker.

Laemmli Buffer (reducing)

31.2% glycerol
6% SDS
1M Tris pH 7.5
872.4mM β-mercaptoethanol
ddH₂O up to 20µl

Laemmli Buffer (non-reducing)

31.2% glycerol
6% SDS
1M Tris pH 7.5
ddH₂O up to 20µl

2.3.2.2 Western Blot

After SDS-PAGE the separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra for optimized protein transfer, Amersham Bioscience). This was performed in a peQlab PerfectBlue “Semi-Dry” Electro Blotter. The blot was prepared as follows:

3 Whatman papers soaked in 1× Transfer buffer were applied to the blotting unit, followed by the nitrocellulose membrane, the gel and finally 3 more wet Whatman papers. To avoid air bubbles a Falcon tube was rolled over the blot. The blot was covered with the lid of the blotting unit. Blotting was performed at 200mA per gel for 1 hour at room temperature. To check transfer efficiency, the membrane was stained with Ponceau S for 1min. Blotted proteins and the standards were visualized by destaining with H₂Odd. The lanes of the loaded samples were marked with a pencil

and excess parts of the membrane were cut off. The membrane was blocked for 90min at 4°C with 5% nonfat dry milk in 1 × TBS-T (Tris-Buffer-Saline, 0,1%Tween) solution. After removing the blocking solution, the primary antibody was also diluted (different dilutions) in blocking solution and the membrane was incubated with this solution overnight at 4°C while gently shaking. The membrane was washed 3× 5-10 minutes in 1× TBS-T, followed by incubation with secondary antibody, which was conjugated with horse raddish peroxidase, diluted (1:50 000) in blocking solution. After 1-2 hours of incubation at room temperature under gentle shaking, the solution was discarded and the membrane was washed another three times with 1× TBS-T for 5-10 minutes. Pierce Super Signal Kit was used to visualize the proteins bound by the secondary antibody. The membrane was incubated with an 1:1 mixture of solution I and II for 2 minutes at room temperature and put into an exposure cassette. Films were exposed in the dark room from 1 min to 1 hour.

2.3.2.3 Coomassie staining

After SDS-PAGE the separation gel was place in a well and Coomassie Blue stain was added until the gel was completely covered by it. The gel was then incubated for 45min. Then, the Coomassie Blue stain was recycled and destaining solution was added to the gel. After 2 hours of incubation, protein bands were well detectable and distinguishable, and the gel was put onto a Whatman paper and dried at 80°C for 30min under vacuum. After drying the gel was analyzed.

1× Transfer Buffer

25mM Tris-HCl
192mM Glycine

10× TBS

1.37M NaCl
0.027M KCl
0.25M Tris-HCl
with HCl to pH 7.4

Ponceau S

0.2% Ponceau S
3% Trichloroacetic Acid
dilute with 1× TBS

1× TBS-T

0.1% Tween
1× TBS
pH 7.4

Coomassie Blue

300ml glacial acetic acid
750ml isopropanol
0.862g Coomassie Blue R250
1950ml ddH₂O

Destaining Buffer

10% acetic acid
30% methanol
ddH₂O up to 1l

2.4 Generation of a Specific Antibody against ggSRBI and ggSRBII

2.4.1 Recombinant Peptide Expression

The PCR product from laying hen chicken liver cDNA with the primer set ggSRBI-Fwd1-NcoI and ggSRBI-Rev1-EcoRI was cloned into the vector pET-25b(+) and chemically competent E.Coli BL-21 were transformed with that plasmid. These transformed bacterias were then used for expression of the recombinant peptide encoded by the ggSRBI DNA sequence amplified by the primer set mentioned above. After purification the recombinant peptide was then used to raise a specific antibody against ggSRBI and ggSRBII by immunization of a rabbit. This recombinant peptide consists of the encoded ggSRBI fragment, a linker amino acid region and an HSV- as well as a 6xHistidine tag. The latter is used for peptide purification. Before recombinant peptide could be used for immunization, it was necessary to check if induction of peptide expression was successful and which buffer would solubilize the peptide best. Therefore two test expressions, namely a time course for induction and a solubilization test, were done with less volume than the final mixture used for purification of enough quantity of recombinant peptide for further use in immunization.

2.4.2 Induction Time Course

A single colony from an LB-amp plate of transformed BL-21 bacterial cells was picked and used for inoculation of 5ml LB-amp medium. This culture was then incubated overnight at 37°C, shaking. 200µl of this starting culture then were transferred into 10ml LB-amp followed by another incubation step at 37°C for 3hours, again shaking. Bacterial cells were allowed to grow to an optical density at 600nm of 0.6, which was measured photometrically. Isopropyl-β-D-thiogalactopyranosid (IPTG)

was added to a final volume of 1mM to induce recombinant peptide expression. Before addition of IPTG, 1.5hours after induction and 3hours after induction samples of 100µl were taken and pipetted into Eppendorf reaction tubes. These samples were centrifuged at 14000rpm for 1min at room temperature and the supernatant was discarded. Pellets were stored at -20°C . The next day these pellets were resuspended in 40µl PBS and two SDS-PAGEs (10% polyacrylamide separation gels) were performed, each being loaded with 10µl of each sample. After SDS-PAGE, one gel was analyzed by Western Blotting and the other one by Coomassie Blue staining

PBS

Sodium Chloride 137mM

Potassium Chloride 137mM

Na_2HPO_4 10mM

KH_2PO_4 1.7mM

2.4.3 Solubilization Test

Pellets of 1ml of recombinant peptide expressing BL-21 cells in LB-amp medium, derived by the procedure described in 2.4.2, were resuspended in 700µl of 4 different types of lysis buffers. TBST, TBST/10mM Imidazol, 5M Urea/PBS Buffer and Lysis Buffer A were used, to check which buffer would be the best for solubilization of the expressed recombinant peptide. The suspensions were sonicated 3 times for 10sec, chilled on ice and then centrifuged at 5000rpm for 10min at 4°C . The supernatant was collected in a new Eppendorf reaction tube and the pellets were resuspended in PBS. 15µl of the suspensions and the supernatant then were analyzed by performing a SDS-PAGE (12%polyacrylamide separating gel), followed by Coomassie Blue staining of the separated proteins. The ratio of recombinant peptide in the pellet fraction and in the supernatant fraction for all lysis buffers was then analyzed. After discussing the result with Professor Dr. Wolfgang J. Schneider, TBST/10mM Imidazol was chosen as lysis buffer for future peptide expressions.

Lysis Buffer A

50mM Tris	0.1% TWEEN 20
500mM NaCl	10% Glycerol
1% Triton X-100	20mM Imidazol

2.4.4 Recombinant Peptide Expression and Purification using Ni-NTA Agarose

Protein expression was done analogous to point 2.4.2, but with more total volume of medium. 7ml overnight starter culture were added to 500ml LB-amp medium. Centrifugation was carried out at 6000rpm for 15min at 4°C. Due to the size of the centrifugation tubes it was necessary to portion the 500ml bacterial culture into two tubes, each 250ml of volume, before centrifugation. Pellet 2 was stored at -20°C as a reserve, while pellet 1 was resuspended in 6ml TBST/10mM Imidazol, except for a small piece of this pellet, which was stored at -20°C as control. The suspension was sonicated 3 times each for 30sec, chilled on ice and centrifuged at 3500rpm for 10min at 4°C. The supernatant was transferred into a 50ml Falcon tube, 1ml Ni-NTA Agarose (QIAGEN) and TBST/10mM Imidazol were added to a final volume of 40ml. The solution was then incubated overnight at 4°C rotating, so Ni-NTA Agarose could bind to soluble recombinant peptide by attaching to the 6xHis tag. The next day the solution was centrifuged at 3500rpm for 5min at 4°C. The supernatant was kept as control and the pellet was washed 4 times with TBST/10mM Imidazol. In between the wash steps the sample was centrifuged at 3500rpm for 5min at 4°C and the supernatants were kept also as controls. After the final wash step the pellet was resuspended in 1ml TBST/10mM Imidazol, transferred into an Eppendorf reaction tube and centrifuged at 6000rpm for 5min at 4°C. The supernatant was discarded and the pellet resuspended in 1ml TBST/250mM Imidazol to elute the recombinant peptide from the Ni-NTA Agarose. The solution was incubated at 4°C for 1hour and 30min rotating, followed by another centrifugation at 6000rpm for 5min at 4°C. The supernatant named Eluate 1, which should contain the recombinant peptide, was transferred into a new Eppendorf reaction tube and the pellet was kept as a control. Efficiency of peptide purification was analyzed by SDS-PAGE under reducing conditions, followed by Coomassie Blue staining of the gel. Eluate 1 was stored at -20°C. The pellet containing the Ni-NTA Agarose was stored at 4°C and the next day another 200µl of TBST/250mM Imidazol were added. The pellet was resuspended

and incubated at 4°C for 2 hours and 30 min, rotating. The suspension was then centrifuged at 6000 rpm for 5 min at 4°C, the supernatant named Eluate 2 was stored at -20°C and the pellet was kept at 4°C.

The day after the pellet was resuspended in 500 µl TBST, 250 mM Imidazole, 2 mM KCL, 25 mM Tris, 300 mM NaCl and was incubated for 2 hours and 30 min at 4°C, rotating. The suspension was then centrifuged again at 6000 rpm for 5 min at 4°C and the supernatant named Eluate 3, was transferred to an Eppendorf reaction tube. These last 3 steps were done one more time. The supernatant derived from the second centrifugation was named Eluate 4 and both supernatants were combined as Eluate 3/4 and stored at -20°C. The pellet was kept at 4°C.

The next day Eluates 1-3/4 and the pellet containing the Ni-NTA agarose bound to the rest of the recombinant peptide were then analyzed by two SDS-PAGEs under reducing and non-reducing conditions, followed by Western Blotting and Coomassie Blue staining. 20 µl from each Eluate and 15 µl of the pellet were loaded. The pellet was stored at 4°C all Eluates at -20°C.

One week later, the pellet was washed 4 times with 10 ml ddH₂O. In between the washing steps the sample was centrifuged at 3500 rpm for 5 min at 4°C. The supernatants were kept as controls. After the final wash step 550 µl of 0.3 M ammonium hydroxide were added to completely clean the Ni-NTA Agarose and remove all recombinant peptide still bound. The sample was vortexed for 1 min and then centrifuged at 6000 rpm for 5 min at 4°C. The supernatant was portioned into two Eppendorf reaction tubes and was lyophilized in a speedvac overnight at 4°C. The pellet was kept at 4°C as a control. The next day the lyophilized samples named Eluate 5/I and 5/II were resuspended in 100 µl reducing Laemmli buffer and stored at -20°C. The day after Eluate 5/I, 5/II and the pellet were then analyzed by two SDS-PAGEs under reducing conditions, followed by Western Blotting and Coomassie Blue staining. All solutions contained protease inhibitor cocktail.

2.4.5 Immunization

The bacterial pellet containing the expressed recombinant peptide that had been stored at -20°C and was not used for purification with Ni-NTA Agarose kit (see 2.4.4), was resuspended in 6ml TBST containing protease inhibitor cocktail and aliquoted into 1.5ml Eppendorf reaction tubes. 4 SDS-PAGEs were done under reducing conditions, 200 μl of bacterial suspension were loaded onto each gel. The separated proteins were Coomassie Blue stained and destained overnight. The next day the band representing the recombinant peptide at a size of $\sim 30\text{kDa}$ was excised with a scalpel, cut into pieces, frozen in liquid nitrogen, ground with a pestle and finally lyophilized overnight at -55°C and 0.03 bar. The next day 400 μl PBS were added and the lyophilized pieces were resuspended. The suspensions were kept at 4°C until they were given to Ao.Univ.-Professor Dr. Marcela Hermann, who then added the same volume Freud's Adjuvans and injected rabbit 75/07 with it. This procedure was repeated after 3 and a half week, 5 weeks and 8 weeks.

15ml blood were taken from the rabbit before immunization (to produce preimmune serum), used as negative control, 15ml 6 weeks after immunization, 50ml after 7 weeks, 15ml after 9 weeks and finally 100ml after 11 weeks. Blood samples were stored at 4°C overnight and centrifuged the next day at 4000rpm for 15min at 4°C . The supernatant immune serum was aliquoted into tubes and stored at -20°C . Binding specificity of the antiserum, crossreactions and dilution factor of the antiserum necessary for adequate results were tested by various Western Blot experiments under reducing and nonreducing conditions, performed with total and membrane protein extracts of laying hens and other species, as well as with the recombinant peptide itself. Antiserum used for Western Blot analysis was diluted in 5% non-fat dry milk, TBST and 5% non-fat dry milk, TBST, 2mM CaCl_2 . Different dilutions were tested. Preimmune serum was used as negative control, diluted 1:100 in 5% non-fat dry milk, TBST.

3 Results

3.1 Cloning of ggSRBI

ggSRBI mRNA sequence was derived from ENSEMBL Genome Browser (Transcript ID GENSscan00000043265, ggSCRBI). The first step of this diploma work was cloning full length ggSRBI and verifying the nucleotide sequence derived from ENSEMBL chicken database. Therefore total RNA was isolated from laying hen liver, in which SRBI expression is highest in humans, using NucleoSpin RNA II kit. Total RNA then was transcribed into cDNA, using oligo-dT primers and SUPERScript II reverse transcriptase. Primers were designed according to the ENSEMBL derived nucleotide sequence and used for PCR of chicken liver cDNA samples mentioned above. Using primerset ggSRBI-F1 and ggSRBI-Rev5 two PCR products of distinct size were obtained (Fig. 3.1.1), depending on the concentration of ggSRBI-F1. GgSRBI-F1 was used at a final concentration of 1 μ M, as well as 1nM, whereas ggSRBI-Rev5 was known to be most efficient at a concentration of 25nM per PCR approach.

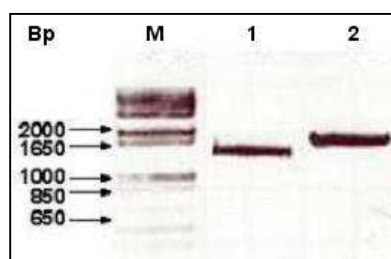


Fig 3.1.1

PCR of chicken liver cDNA using primerset ggSRBI-F1/Rev5.

1... 1 μ M ggSRBI-F1

2... 1nM ggSRBI-F1

Both PCR products were cloned into pCR 2.1-Topo, using a Topo TA cloning kit. Plasmid minipreparation followed by EcoRV restriction enzyme digestion confirmed PCR results.

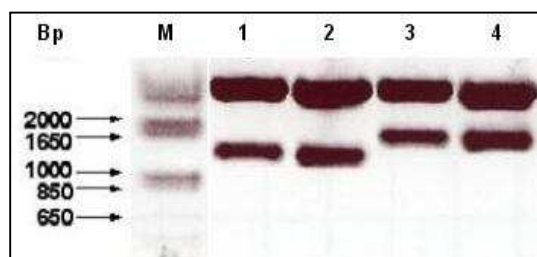


Fig. 3.1.2

EcoRV restriction enzyme digestion

1,2... 1 μ M ggSRBI-F1

3,4... 1nM ggSRBI-F1

Results

Sequencing of inserts 2 and 4 revealed there is no difference between the nucleotide sequence of insert 2 and the ENSEMBL sequence of ggSRBI (Fig. 3.1.3), concluding Insert 2 corresponds to full length ggSRBI.

Insert 4 on the other hand showed no similarity with that sequence. Lower concentration of ggSRBI-F1, in combination with the PCR program used, seem to result in amplification of a completely different, unspecific PCR Product.

ggSRBI_Ensembl	-----ATG	GTCTGCGCGCCGGGCGCAGGGTGGCGGTGGGGCTGGGGCGGTCTG	46
F1-ggSRBI-Rev5	C CGCTCTGCGAGCC	ATGTCGCGCGCCGGGCGCAGGGTGGCGGTGGGGCTGGGGCGGTCTG	60
	→	*****	
ggSRBI_Ensembl	GGGCCGTTTGCCTGCTGCTGGGCGGCTGCCTGCTGCTGGCGGGGCCGCCATCGTCAAGG		106
F1-ggSRBI-Rev5	GGGCCGTTTGCCTGCTGCTGGGCGGCTGCCTGCTGCTGGCGGGGCCGCCATCGTCAAGG		120

ggSRBI_Ensembl	AGCAGGTCTCAAGAATGTGAGGATTGACCCAGCAGCATCTCCTTCAACATGTGGAGGG		166
F1-ggSRBI-Rev5	AGCAGGTCTCAAGAATGTGAGGATTGACCCAGCAGCATCTCCTTCAACATGTGGAGGG		180

ggSRBI_Ensembl	ACATCCCTGTTCTCTTTTATCTGACGGTGAACCTCTTTGAAGTGCAGAACCCCAAGAGG		226
F1-ggSRBI-Rev5	ACATCCCTGTTCTCTTTTATCTGACGGTGAACCTCTTTGAAGTGCAGAACCCCAAGAGG		240

ggSRBI_Ensembl	TCCTCCAGGGAGCGAAGCCAAAGGTGAACCAGCGTGGACCGTATGTTTACAGAGAGTTCA		286
F1-ggSRBI-Rev5	TCCTCCAGGGAGCGAAGCCAAAGGTGAACCAGCGTGGACCGTATGTTTACAGAGAGTTCA		300

ggSRBI_Ensembl	AGTATAAGACAAACATCACTTTCCATGACAACGACACCGTCTCTTACCTGGAGTACCGGC		346
F1-ggSRBI-Rev5	AGTATAAGACAAACATCACTTTCCATGACAACGACACCGTCTCTTACCTGGAGTACCGGC		360

ggSRBI_Ensembl	AGCTCTTCTTCCGGCCAGACTTGTCCAACGGCACTGAAGATGAGTACATCGTGATGCCCA		406
F1-ggSRBI-Rev5	AGCTCTTCTTCCGGCCAGACTTGTCCAACGGCACTGAAGATGAGTACATCGTGATGCCCA		420

ggSRBI_Ensembl	ACATCTTGATGTTGGGAGCAGCTGTCATGATGGAAAACCTGCCTAGTTTTGTGAAGCTCT		466
F1-ggSRBI-Rev5	ACATCTTGATGTTGGGAGCAGCTGTCATGATGGAAAACCTGCCTAGTTTTGTGAAGCTCT		480

ggSRBI_Ensembl	TGCTGAGCGGGCCCTGGCTGGCCTGAAGCAGGAGGCCTTCATAAACCGGACAGTGGGGG		526
F1-ggSRBI-Rev5	TGCTGAGCGGGCCCTGGCTGGCCTGAAGCAGGAGGCCTTCATAAACCGGACAGTGGGGG		540

ggSRBI_Ensembl	AGATCTTGTGGGGCTATGAAGACCCCTCTTTTAGACACCATAAATGCCCTTGTCCTGGCC		586
F1-ggSRBI-Rev5	AGATCTTGTGGGGCTATGAAGACCCCTCTTTTAGACACCATAAATGCCCTTGTCCTGGCC		600

ggSRBI_Ensembl	TGATCCCATAACAAGGGGAAATTGGCATATTTATAGAGTTTAAACAACACCAATTCTGGAT		646
F1-ggSRBI-Rev5	TGATCCCATAACAAGGGGAAATTGGCATATTTATAGAGTTTAAACAACACCAATTCTGGAT		660

ggSRBI_Ensembl	TGTTACAGTGAACACAGGCATGAAGAACATCAGTCAAGTTCATATGGTGGATTTCATGGA		706
F1-ggSRBI-Rev5	TGTTACAGTGAACACAGGCATGAAGAACATCAGTCAAGTTCATATGGTGGATTTCATGGA		720

ggSRBI_Ensembl	ATGGCTTGAAAAAGGTGAACTACTGGCGGACCAATGAGTGCAACATGATCAATGGGACTG		766
F1-ggSRBI-Rev5	ATGGCTTGAAAAAGGTGAACTACTGGCGGACCAATGAGTGCAACATGATCAATGGGACTG		780

ggSRBI_Ensembl	CAGGGGAGATGTGGCCACCGTATATGTCCCAACATCGCTGGAGTTCTACAGCCCCGATG		826
F1-ggSRBI-Rev5	CAGGGGAGATGTGGCCACCGTATATGTCCCAACATCGCTGGAGTTCTACAGCCCCGATG		840

ggSRBI_Ensembl	CCTGCAGATCCATGACACTGGTGTACGAGCAGTCGGGGAAATTCAAGGGTGTGCCACCT		886
F1-ggSRBI-Rev5	CCTGCAGATCCATGACACTGGTGTACGAGCAGTCGGGGAAATTCAAGGGTGTGCCACCT		900

ggSRBI_Ensembl	ATCGCTTTGTGGCACCAGAAACTCTCTTTGCCAATGGCACAGATTATCCCCCAACGAAG		946
F1-ggSRBI-Rev5	ATCGCTTTGTGGCACCAGAAACTCTCTTTGCCAATGGCACAGATTATCCCCCAACGAAG		960

ggSRBI_Ensembl	GCTTCTGTCCCTGCAGGCAATCCGGCATCCAGAACGTCAGCTCCTGCAGGCTGAATGCAC	1006
F1-ggSRBI-Rev5	GCTTCTGTCCCTGCAGGCAATCCGGCATCCAGAACGTCAGCTCCTGCAGGCTGAATGCAC	1020

ggSRBI_Ensembl	CAATGTTTCATCTCCCACCTCACTTCTACAATGCTGACCCAAGCCTGGTGGATGCTGTGG	1066
F1-ggSRBI-Rev5	CAATGTTTCATCTCCCACCTCACTTCTACAATGCTGACCCAAGCCTGGTGGATGCTGTGG	1080

ggSRBI_Ensembl	AAGGCCTGCACCCAGCAGGGAGGAGCACGCGCTCTTCCTCGATGTGCACCCGGTGACAG	1126
F1-ggSRBI-Rev5	AAGGCCTGCACCCAGCAGGGAGGAGCACGCGCTCTTCCTCGATGTGCACCCGGTGACAG	1140

ggSRBI_Ensembl	GCATCCCCATGAAGTGTCTCCATCAAGTCCAGCTGAACAGTACATGAAGCAGGTGTCTG	1186
F1-ggSRBI-Rev5	GCATCCCCATGAAGTGTCTCCATCAAGTCCAGCTGAACAGTACATGAAGCAGGTGTCTG	1200

ggSRBI_Ensembl	GTATCCTTCAAACAGGGAAGATTGAGCCTGTGGTGATGCCGCTGCTGTGGTTTGCAGAGA	1246
F1-ggSRBI-Rev5	GTATCCTTCAAACAGGGAAGATTGAGCCTGTGGTGATGCCGCTGCTGTGGTTTGCAGAGA	1260

ggSRBI_Ensembl	GTGGATACATTGATGGCTCAGTCCTATCGCAGTTTTACACCAACCTGGTGCTGATCCCAT	1306
F1-ggSRBI-Rev5	GTGGATACATTGATGGCTCAGTCCTATCGCAGTTTTACACCAACCTGGTGCTGATCCCAT	1320

ggSRBI_Ensembl	CCATCCTAGACTACCTGCAGTACATCTTCCTCGGGCTCAGCGTCCCGCTCATCATCTCAG	1366
F1-ggSRBI-Rev5	CCATCCTAGACTACCTGCAGTACATCTTCCTCGGGCTCAGCGTCCCGCTCATCATCTCAG	1380

ggSRBI_Ensembl	CTGCACTCGTGATGATGAGTCAGAAGTCCTCCAAGGAGAAGCACCCACACGACCCACCA	1426
F1-ggSRBI-Rev5	CTGCACTCGTGATGATGAGTCAGAAGTCCTCCAAGGAGAAGCACCCACACGACCCACCA	1440

ggSRBI_Ensembl	CGCAGAGCCCCCACCACAGCCCTCCGAGACCACACCGCTCCTCCAGGACACCGGCACAC	1486
F1-ggSRBI-Rev5	CGCAGAGCCCCCACCACAGCCCTCCGAGACCACACCGCTCCTCCAGGACACCGGCACAC	1500

ggSRBI_Ensembl	AGAGCCAGGACACCGCCGAGGCTGA-----	1512
F1-ggSRBI-Rev5	AGAGCCAGGACACCGCCGAGGCTGAGCTTGGGATGGGAAGTCTTCAGATCACCACCAG	1560

ggSRBI_Ensembl	-----	
F1-qgSRBI-Rev5	TCTCCAGAAGGGACGTGTC	1580

Fig. 3.1.3 Alignment of ggSRBI nucleotide sequence derived from ENSEMBL and PCR of chicken liver cDNA using primerset ggSRBI-F1/Rev5.

Red...Primers

Blue...Start and stop of translation

The PCR product of ggSRBI-F1/Rev5 is of a length of 1580bp and contains an 1512bp open reading frame, coding for ggSRBI protein (Fig.3.1.4).

1	M	V	A	A	G	R	R	V	A	V	G	L	G	A	V	G	A	V	C	L	20																	
1	a	t	g	g	t	c	g	c	g	g	c	g	c	a	g	g	t	g	g	c	g	g	t	c	g	g	c	c	t	t	g	c	c	t	g	60		
21	L	L	G	G	C	L	L	L	A	G	P	A	I	V	K	E	Q	V	L	K	40																	
61	c	t	g	c	t	g	g	c	g	g	c	t	g	c	t	g	c	t	g	g	c	g	g	c	c	c	c	c	c	c	c	c	c	a	g	120		
41	N	V	R	I	D	P	S	S	I	S	F	N	M	W	R	D	I	P	V	P	60																	
121	a	a	t	g	t	g	a	g	a	t	t	g	a	c	c	c	a	c	a	c	a	t	c	t	c	t	t	c	a	a	c	a	t	g	t	g	180	
61	F	Y	L	T	V	N	L	F	E	V	Q	N	P	Q	E	V	L	Q	G	A	80																	
181	t	t	t	a	t	c	t	g	a	c	c	t	c	t	t	t	g	a	a	g	t	g	c	a	a	c	c	c	c	a	a	g	a	g	t	c	c	240
81	K	P	K	V	N	Q	R	G	P	Y	V	Y	R	E	F	K	Y	K	T	N	100																	
241	a	a	g	c	c	a	a	g	t	g	a	a	c	c	a	c	a	c	a	c	a	c	c	a	c	a	c	a	a	a	a	a	a	a	a	300		

Results

```

101 I T F H D N D T V S Y L E Y R Q L F F R 120
301 atcactttccatgacaacgacaccgtctcctacctggagtaccggcagctcttctccgg 360

121 P D L S N G T E D E Y I V M P N I L M L 140
361 ccgagacttgccaacggcactgaagatgagtacatcgatgcccaacatcttgatgttg 420

141 G A A V M M E N L P S F V K L L L S G A 160
421 ggagcagctgtcatgatggaaaacctgcctagttttgtgaagctcttgctgagcggggcc 480

161 L A G L K Q E A F I N R T V G E I L W G 180
481 ctggctggcctgaagcaggaggccttcataaaccggacagtgggggagatcttggtgggc 540

181 Y E D P L L D T I N A L V P G L I P Y K 200
541 tatgaagaccctcttttagaccataaatgcccttgccctggcctgatcccatacaag 600

201 G K F G I F I E F N N T N S G L F T V N 220
601 gggaaatttggcatatttatagagttaaacaacaccaattctggattgttcacagtgaac 660

221 T G M K N I S Q V H M V D S W N G L K K 240
661 acaggcatgaagaacatcagtcagttcatatggtggattcatggaatggcttgaaaaag 720

241 V N Y W R T N E C N M I N G T A G E M W 260
721 gtgaactactggcggaccaatgagtgcacatgatcaatgggactgcaggggagatgtgg 780

261 P P Y M S P T S L E F Y S P D A C R S M 280
781 ccaccgtatatgtcccaacatcgctggagttctacagccccgatgcctgcagatccatg 840

281 T L V Y E Q S G K F K G V P T Y R F V A 300
841 aactgggtgtacgagcagtcggggaaattcaagggtgtgccacatcgtcttgggca 900

301 P K T L F A N G T D Y P P N E G F C P C 320
901 ccgaaaactctctttgccaatggcacagattatcccccaacgaaggcttctgtccctgc 960

321 R Q S G I Q N V S S C R L N A P M F I S 340
961 aggcaatccggcatccagaacgtcagctcctgcagggtgaatgcaccaatgttcatctcc 1020

341 H P H F Y N A D P S L V D A V E G L H P 360
1021 caccctcacttctacaatgctgaccaagcctggtggatgctgtggaaggcctgcacccc 1080

361 S R E E H A L F L D V H P V T G I P M N 380
1081 agcagggaggagcacgcgctcttctcgcgatgtgcacccggtgacaggcatcccatgaac 1140

381 C S I K L Q L N Q Y M K Q V S G I L Q T 400
1141 tgtcccatcaagctccagctgaaccagttacatgaagcaggtgtctggtatccttcaaaca 1200

401 G K I Q P V V M P L L W F A E S G Y I D 420
1201 ggggaagattcagcctgtggtgatgccgctgctgtggtttgcagagagtgatacatgtat 1260

421 G S V L S Q F Y T N L V L I P S I L D Y 440
1261 ggctcagtcctatcgagttttacaccaacctggtgctgatcccatccatcctagactac 1320

441 L Q Y I F L G L S V P L I I S A A L V M 460
1321 ctgcagtagatcttctcgggctcagcgtcccgcctcatctcagctgcactcgtgatg 1380

461 M S Q K S S K E K H P H D P T T Q S P P 480
1381 atgagtcagaagtcctccaaggagaagcaccacacgacccaccacgcagagcccccca 1440

481 P Q P S E T T P L L Q D T G T Q S Q D T 500
1441 cccagccctccgagaccacaccgctcctccaggacaccggcacacagagccaggacacc 1500

501 A E A * 504
1501 gccgaggcctga 1512

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Fig. 3.1.4 Alignment of ggSRBI cDNA nucleotide- and ggSRBI protein amino acid sequence.

3.2 Gene Expression of ggSRBI at the mRNA Level

For investigation of genexpression and tissue distribution of ggSRBI at the mRNA level, various Northern blots were performed. According to ENSEMBL database ggSRBI mRNA consists of 1821 nucleotides. The PCR products of primer sets ggSRBI-fwd4/Rev4 (1330bp) and ggSRBI-Fwd1/Rev1 (704bp) (Fig. 3.2.1) were used as radiolabelled probes. For optimization of amplification output plasmid minipreparations of ggSRBI-fwd4/Rev4 and ggSRBI-Fwd1/Rev1, derived from RT-PCR of laying hen liver total RNA, cloned into pCR 2.1-Topo were used as template. For exclusion of PCR derived sequence errors, these minipreparations were sequenced before being used as templates.

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atgggtcgcgccggcgccgaggggtggcggtggggctggggcggtcggggcggtttgctgctgctggcgggctgcctgct 80
gctggcgggggcccgccatcgctcaaggagcaggtcctcaagaatgtgaggattgacccagcagcgcacatccttcaacatgt 160
ggagggacatccctgttctcttttatctgacggtgaacctctttgaagtgcagaaccccccaagaggtcctccaggagcgc 240
aagccaaagggtgaaccagcgtggaccgtatgtttacagagagtccaagtataagacaaacatcactttccatgacaacga 320
caccgtctcctacctggagtaccggcagctcttcttcggccagacttgccaacggcactgaagatgagtacatcgtga 400
tgcccaacatcttgatggtgggagcagctgtcatgatggaaaacctgcctagttttgtgaagctcttgctgagcggggcc 480
ctggctggcctgaagcaggagggccttcataaacggacagtgggggagatcttggtgggctatgaagacctctctttaga 560
caccataaatgcccttgctcctggcctgatccatacaaggggaaatttggcatatttatagagtttaacaacaccaatt 640
ctggattgttcacagtgaacacagggcatgaagaacatcagtcgaagttcatatgggtgattcatggaatggccttgaaaaag 720
gtgaactactggcggaaccaatgagtgcaacatgatcaatgggactgcaggggagatgtggccaccgtatatgtccccaac 800
atcgctggagttctacagcccgatgcctgcagatccatgacactggtgtacgagcagtcggggaaattcaaggggtgtgc 880
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gggaagattcagcctgtggtgatgccgtgctgtggtttgcagagagtggtacattgatggctcagtcctatcgagtt 1280
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tctcagctgcactcgtgatgatgagtcagaagtcctccaaggagaagcaccacacgagccaccacgcagagccccca 1440
ccccagcctccgagaccacacgcgtcctccaggacaccggcacacagagccaggacaccgcgagggcctga 1512

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Fig. 3.2.1 Nucleotide sequence of ggSRBI. Primer sets used for generation of radiolabelled probes for Northern blot analysis are marked in green (ggSRBI-Fwd4/Rev4) and red (ggSRBI-Fwd1/Rev1).

Before performing Northern blots for all chicken tissues, functionality of the probes used had to be tested. Thus, only tissues known to show high expression levels of ggSRBI mRNA were used. Total RNA was isolated from laying hen chicken tissues, using TRI reagent. The first Northern blot approach was performed for each 30µg of laying hen liver and small intestine total RNA. Methylene blue staining of the membrane (Fig. 3.2.2) served as loading- and quality of RNA control.

Results

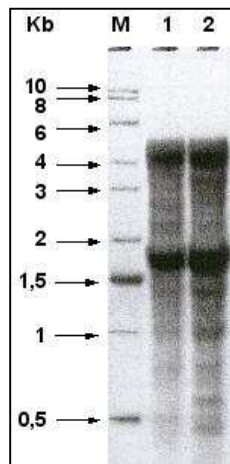


Fig. 3.2.1

Methylene blue staining of Northern blot membrane

1...30µg liver total RNA

2...30µg small intestine total RNA

Radiolabelled PCR product of ggSRBI-Fwd4/Rev4 was used as hybridization probe and films were exposed to the membrane for 1day (d), 2d and 4d (Fig. 3.2.3).

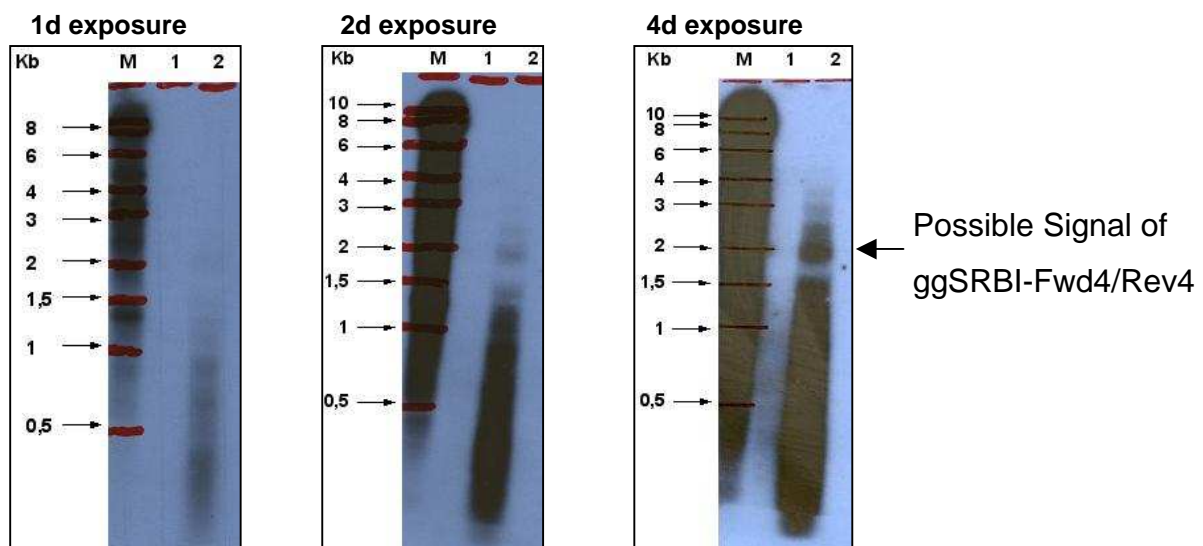


Fig. 3.2.2 *Northern Blot of laying hen tissues total RNA, using PCR product of ggSRBI-Fwd4/Rev4 as probe. 1...Liver, 2...Small Intestine*

Hybridization did not work properly. Most of the probe seemed to have bound to the marker lane, whereas there was no signal detectable in liver, even though SRBI expression levels are supposed to be highest in liver. In small intestine a signal was detected, corresponding to the size of gSRBI predicted by ENSEMBL, but still there occurred plenty of unspecific binding, so no certain conclusion could be drawn. Probably the SRBI RNA was degraded. Thus new RNA was isolated from laying hen liver and adrenals, which are known to show the highest expression levels of SRBI per gram tissue, and another Northern blot was performed.

This time, 16µg total RNA of each tissue were applied for Northern blotting. Again PCR product of ggSRBI-Fwd4/Rev4 was used as radiolabelled probe, but also a positive control was introduced, using a chicken-apoAV probe, kindly donated by Dr. Andrea Dichlberger. If hybridization worked out, apoAV mRNA should become visible as double band at 2300kb and 1430kb. Total RNAs for both approaches were loaded onto an agarose gel, run together, transferred to the nylon membrane and methylene blue stained (Fig. 3.2.3).

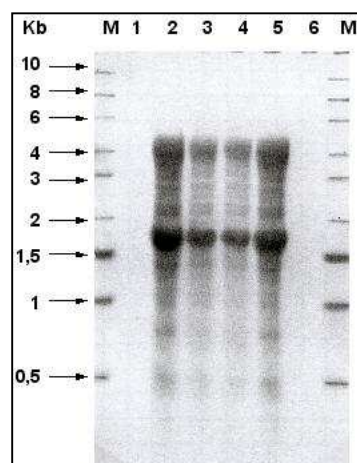


Fig. 3.2.3

Methylene blue staining of Northern blot membrane

1,6...empty

2,5...16µg liver total RNA

3,4...16µg adrenals total RNA

After staining the membrane was cut into two pieces, each consisting of a marker-, liver- and adrenals total RNA lane. These two membrane pieces then were analogly treated for hybridization, using the apoAV or the ggSRBI probe (3.2.4) and films were exposed to the membranes for 2d and 3d.

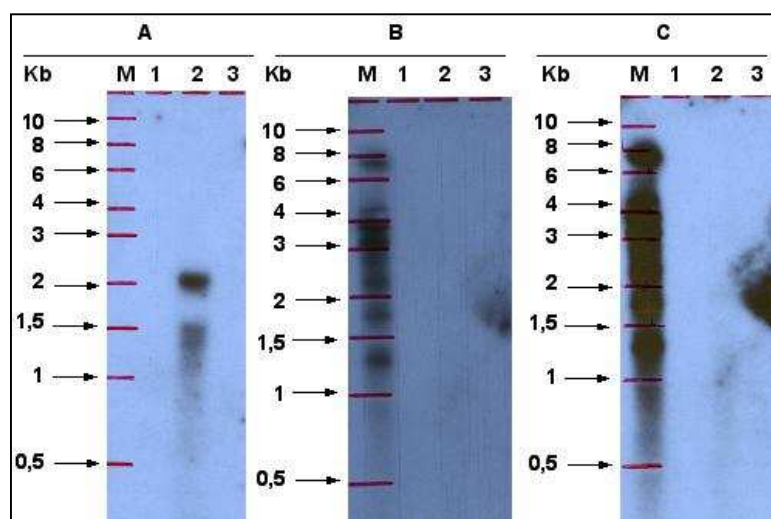


Fig. 3.2.4 *Northern Blot of laying hen chicken total RNA, using PCR product of ggSRBI-Fwd4/Rev4 as probe and apoAV as positive control. 1...Empty, 2...Liver, 3...Adrenals*

A...apoAV / 3d exposure, B...ggSRBI/ 2d exposure, C...ggSRBI/3d exposure

Results

The positive control worked very well. Two distinct signals were detected in liver total RNA corresponding to the predicted size of apoAV mRNA (2300kb, 1821kb). The signal at ~2300 was very sharp, whereas the signal at ~1800kb was more smudgy, consistent with former experiments of Dr. Andrea Dichlberger. As the positive control resulted in detectable signals, RNA was of proper quality and no methodological error had occurred. Also no radiolabelled probe had bound to the marker lane. But in case of ggSRBI-Fwd4/Rev4, again most of the probe was bound by the marker and there was neither a specific signal in adrenals nor in liver.

As the problem had not been RNA quality and probe-binding of the marker seemed to be probe specific, therefore another Northern blot approach was performed, including PCR products of ggSRBI-Fwd4/Rev4, as well as ggSRBI-Fwd1/Rev1 as radiolabelled probes for ggSRBI mRNA detection and again apoAV as positive control.

24,6µg of laying hen liver total RNA per approach were loaded onto the agarose gel. Northern blot analysis was performed as described above. Methylene blue staining again served as loading control (Fig.3.2.6).

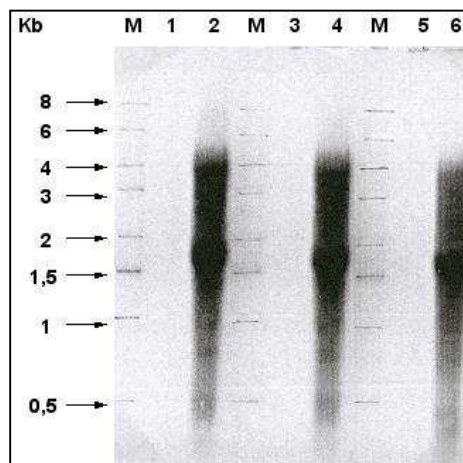


Fig. 3.2.6 *Methylene blue staining of Northern blot membrane*

1,3,5...Empty, 2,4,,6...24,6µg liver total RNA

GgSRBI and apoAV probes were used for hybridization and photosensitive films were exposed for 2d (Fig. 3.2.7)

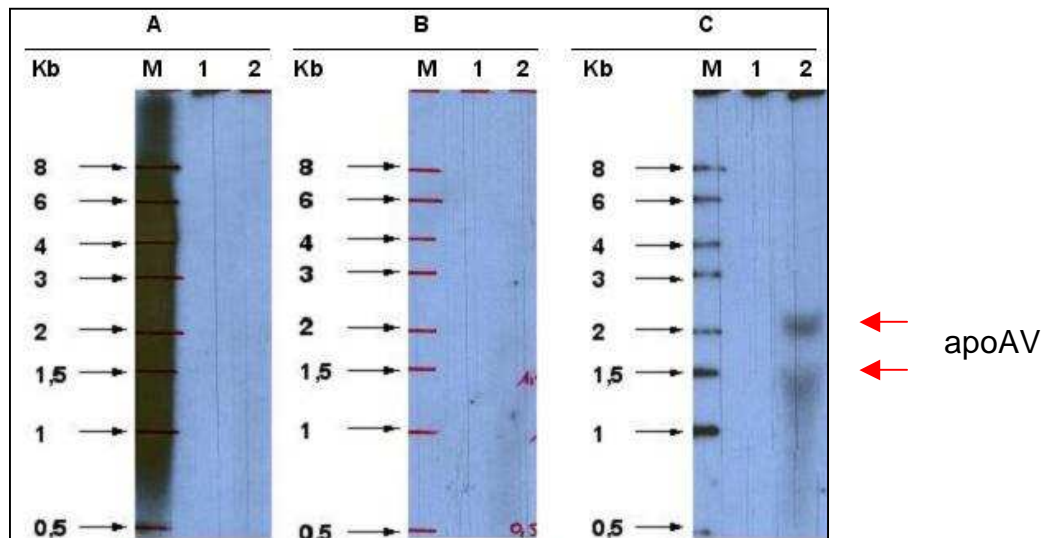


Fig. 3.2.7 Northern Blot of laying hen chicken liver total RNA, using PCR products of ggSRBI-Fwd4/Rev4 and ggSRBI-Fwd1/Rev1 as probe, as well as apoAV as positive control. Films exposed for 2d.

A...ggSRBI-Fwd4/Rev4, B...ggSRBI-Fwd1/Rev1, C...apoAV
1...Empty, 2...Liver

Just like before, positive control worked very well and ggSRBI-Fwd4/Rev4 radiolabelled probe was bound to the marker. No signal was detected in liver total RNA. In contrast ggSRBI-Fwd1/Rev1 radiolabelled probe didn't bind to the marker, but there also was no signal detected in liver. In case of ggSRBI-Fwd1/Rev1, obviously no binding at all had occurred. Therefore the membrane was incubated with radiolabelled ggSRBI-Fwd1/Rev1 again, but under less stringent hybridization conditions. Hybridization temperature was lowered from 65°C to 60°C (Fig. 3.2.8).

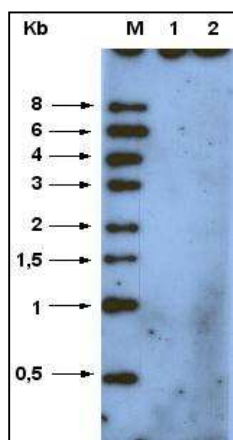


Fig. 3.2.8

Northern Blot of laying hen chicken liver total RNA, using PCR product of ggSRBI-Fwd1/Rev1 as probe. Hybridization was performed at 60°C. Film was exposed to the Northern blot membrane for 5d.

1...Empty
2...Liver

Even though the probe did not bind to the marker, again no signal could be detected in liver.

3.3. Generation of an anti-ggSRBI Peptide Antibody

For further experiments an antibody against ggSRBI was raised. Therefore a fragment of ggSRBI of a length of 704bp (Fig. 3.3.1) was cloned into pET-25b(+), using primer set ggSRBI-Fwd1-NcoI/Rev1-EcoRI for PCR and expressed recombinantly in chemically competent E.Coli. This peptide (Fig. 3.3.2), with a predicted molecular weight of 31,26 kDa then was used for immunization of a rabbit.

ggSRBI	CTGCTGGGCGGCTGCCTGCTGCTGGCGGGGCCGCCATCGTCAAGGAGCAGGTCCTCAAG	120
NcoI-Fwd1/Rev1-EcoRI	-----TACCATG	7

ggSRBI	AATGTGAGGATTGACCCAGCAGCATCTCCTTCAACATGTGGAGGGACATCCCTGTTCCCT	180
NcoI-Fwd1/Rev1-EcoRI	GATGTGAGGATTGACCCAGCAGCATCTCCTTCAACATGTGGAGGGACATCCCTGTTCCCT	67

ggSRBI	TTTTATCTGACGGTGAACCTCTTTGAAGTGCAGAACCCCCAAGAGGTCCTCCAGGGAGCG	240
NcoI-Fwd1/Rev1-EcoRI	TTTTATCTGACGGTGAACCTCTTTGAAGTGCAGAACCCCCAAGAGGTCCTCCAGGGAGCG	127

ggSRBI	AAGCCAAAGGTGAACCAGCGTGGACCGTATGTTTACAGAGAGTTCAAGTATAAGACAAAC	300
NcoI-Fwd1/Rev1-EcoRI	AAGCCAAAGGTGAACCAGCGTGGACCGTATGTTTACAGAGAGTTCAAGTATAAGACAAAC	187

ggSRBI	ATCACTTTCCATGACAACGACACCGTCTCCTACCTGGAGTACCGGCAGCTCTTCTTCCGG	360
NcoI-Fwd1/Rev1-EcoRI	ATCACTTTCCATGACAACGACACCGTCTCCTACCTGGAGTACCGGCAGCTCTTCTTCCGG	247

ggSRBI	CCAGACTTGTCCAACGGCACTGAAGATGAGTACATCGTGATGCCCAACATCTTGATGTTG	420
NcoI-Fwd1/Rev1-EcoRI	CCAGACTTGTCCAACGGCACTGAAGATGAGTACATCGTGATGCCCAACATCTTGATGTTG	307

ggSRBI	GGAGCAGCTGTGATGATGGAAAACCTGCCTAGTTTTGTGAAGCTCTTGCTGAGCGGGGCC	480
NcoI-Fwd1/Rev1-EcoRI	GGAGCAGCTGTGATGATGGAAAACCTGCCTAGTTTTGTGAAGCTCTTGCTGAGCGGGGCC	367

ggSRBI	CTGGCTGGCCTGAAGCAGGAGGCCTTCATAAACCGGACAGTGGGGGAGATCTTGTGGGGC	540
NcoI-Fwd1/Rev1-EcoRI	CTGGCTGGCCTGAAGCAGGAGGCCTTCATAAACCGGACAGTGGGGGAGATCTTGTGGGGC	427

ggSRBI	TATGAAGACCTCTTTTAGACACCATAAATGCCCTTGTCCTGGCCTGATCCCATACAAG	600
NcoI-Fwd1/Rev1-EcoRI	TATGAAGACCTCTTTTAGACACCATAAATGCCCTTGTCCTGGCCTGATCCCATACAAG	487

ggSRBI	GGGAAATTTGGCATATTTATAGAGTTTAACAACACCAATTCTGGATTGTTACAGTGAAC	660
NcoI-Fwd1/Rev1-EcoRI	GGGAAATTTGGCATATTTATAGAGTTTAACAACACCAATTCTGGATTGTTACAGTGAAC	547

ggSRBI	ACAGGCATGAAGAACATCAGTCAAGTTCATATGGTGGATTATGGAATGGCTTGAAAAAG	720
NcoI-Fwd1/Rev1-EcoRI	ACAGGCATGAAGAACATCAGTCAAGTTCATATGGTGGATTATGGAATGGCTTGAAAAAG	607

ggSRBI	GTGAACTACTGGCGGACCAATGAGTGCAACATGATCAATGGGACTGCAGGGGAGATGTGG	780
NcoI-Fwd1/Rev1-EcoRI	GTGAACTACTGGCGGACCAATGAGTGCAACATGATCAATGGGACTGCAGGGGAGATGTGG	667

ggSRBI	CCACCGTATATGTCCCAACATCGCTGGAGTTCTACAGCCCCGATGCTGCAGATCCATG	840
NcoI-Fwd1/Rev1-EcoRI	CCACCGTATATGTCCCAACATCGCTGGAGTTCTACAGCCCCGATGCTGCAGATCCATG	725

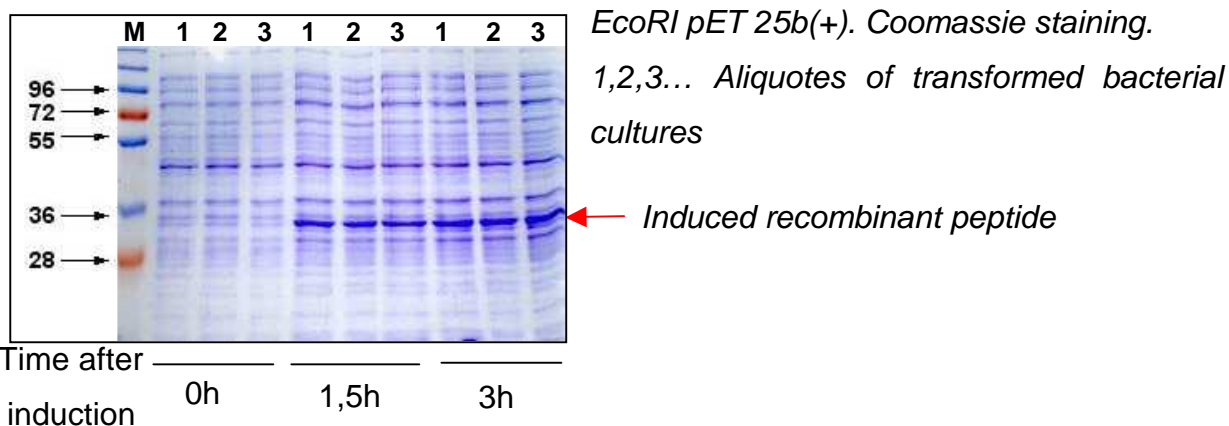
Fig.3.3.1 Nucleotide alignment of ggSRBI cDNA and the PCR product of the primer set ggSRBI-Fwd1-NcoI/Rev1-EcoRI (marked in red).

MDVRIDPSSISFNMWRDIPVPFYLTVNLFVQNPQEV LQGAKPKVNQRGPYVYREFKYKT-
 NITFHDNDTVSYLEYRQLFFRPDL SNGTEDEYIVMPNILMLGAAVMMENLP SFVKLLLSGA-
 LAGLKQEAFINRTVGEILWGYEDPLD TINALVPGLIPYKGKFGI-
 FIEFNNTNSGLFTVNTGMKNISQVHMVDSWNLKKVNYWRTNECNMINGTAGEMWP-
 PYMSPTSLEFYSPDAKNSSSVDKAAALEIKRASEPELAPEDPEDVEHHHHHH

Fig 3.3.2 Amino acid sequence of the recombinant peptide used for immunization. Cloned *ggSRBI* fragment is marked in red (*ggSRBI-Fwd1-ncol/Rev1-EcoRI*), *HSV tag* and *6xHis tag* of *pET-25b(+)* in green and blue. The predicted molecular weight for this peptide is 31,26kDa.

As predicted, induction of *E. Coli*, transformed with the construct mentioned above, resulted in an increase of a peptide with a molecular weight of ~32kDa (Fig. 3.3.3)

Fig. 3.3.3 Induction time course of *E. Coli* transformed with *ggSRBI-Fwd1-ncol/Rev1-*



Purification of the recombinant peptide should be carried out, using Ni-NTA Agarose (Fig. 3.3.4).

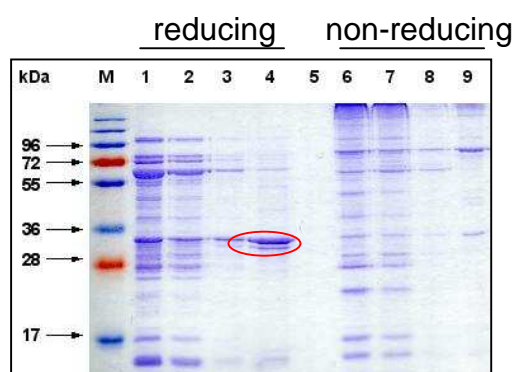


Fig. 3.3.4

Ni-NTA Agarose purification of recombinantly expressed ggSRBI-Fwd1-ncol/Rev1-EcoRI. Coomassie staining. 1/6,2/7,3/8,...Aliquotes of eluats from different elution steps (see 2.4.4); 4,9...Ni-NTA Agarose after all elution steps.

Even multiple elution and wash steps (see 2.4.4) were not able to elute the recombinant peptide from the Ni-NTA Agarose completely. Once bound to it, most of the recombinant peptide stayed within the Agarose. Thus, the peptide was used for immunization without further purification (see 2.4.5). Immune sera of all 4 bleedings of the immunized rabbit were tested by performing Western blots of laying hen chicken liver

Results

total protein extracts, using the sera as primary antibody. According to the ENSEMBL database, ggSRBI has a predicted molecular weight of ~56kDa. Human SRBI has a molecular weight of 57kDa, but is heavily glycosylated and migrates on SDS gels at 82kDa. Glycosylation status of ggSRBI was not known at the time of these experiments. The four sera visualized bands corresponding to a molecular weight of ~64kDa in laying hen liver total protein extracts, which were not present when pre-immune serum was used. (Fig. 3.3.5).

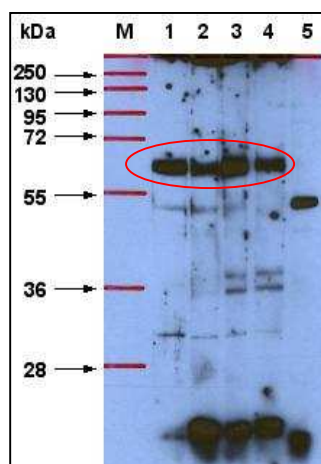


Fig. 3.3.5

Western Blot (reducing conditions, mercaptoethanol), of laying hen liver total protein extracts (45µg per lane), using sera 1-4 as primary antibody diluted 1:500 in blocking solution (TBST, 2mM CaCl₂, 5% milk). Film exposure 2sec.

1...Serum 1; 2...Serum 2

3...Serum 3; 4...Serum 4

5...Pre-immune serum (1:100)

Subsequently serum 4 was tested for detection efficiency at different concentrations (Fig. 3.3.6).

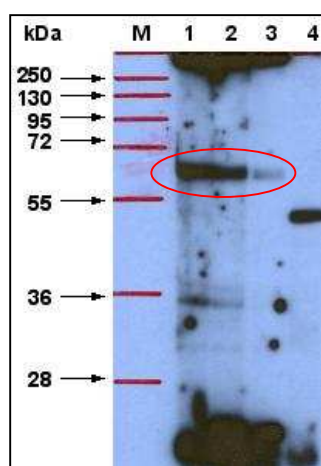


Fig. 3.3.6

Western Blot (reducing conditions, mercaptoethanol), of laying hen liver total protein extracts (45µg per lane), using different dilutions of serum 4 in blocking solution as primary antibody. Film exposure 2sec.

1...1:100

2...1:500

3...1:1000

4...Pre-immune serum (1:100)

Again a band of ~64kDa was detected and according to the dilution factor of the primary antibody, signal intensity changed.

Results of those two experiments indicate immunization did work out very well and immunomactive sera of bleeding 1-4 are suitable for detection of ggSRBI. Therefore these sera were named rabbit (rb) anti-ggSRBI. For further investigations some Western blots were performed under reducing and non-reducing conditions (Fig. 3.3.7)

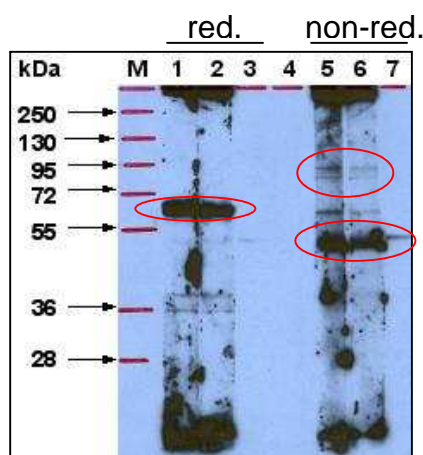


Fig. 3.3.7

Western Blot of laying hen liver total protein extracts (45µg per lane) using *rb-anti-ggSRBI* as primary antibody. Film exposure 10sec.

1,5... *rb-anti-ggSRBI* (1:500)

2,6...*rb-anti-ggSRBI* (1:1000)

3,7...Pre-immune serum (1:500)

4...Empty

Reducing conditions resulted in the same ~64kDa bands as usual, whereas under non-reducing conditions the ~64kDa band almost disappear. Instead bands of other size became visible.

In further Wester blot experiments, so called competition assays (Fig. 3.3.8), 20µl of the solution of the recombinant peptide, used for immunization, were added as competitor to *rb-anti-ggSRBI*. In these experiments recombinant peptide solutions were supposed to compete for total protein extract ggSRBI binding to *rb-anti-ggSRBI*.

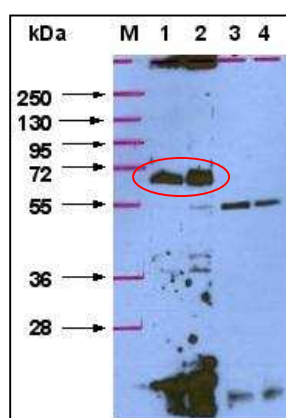


Fig. 3.3.8

Competition assay with *rb-anti-ggSRBI*

45µg laying hen total liver protein extract per lane

Film exposure 20sec

1...*rb-anti-ggSRBI*; -competitor

2...*rb-anti-ggSRBI*; +competitor

3...Pre-immune serum; -competitor

4...Pre-immune serum; +competitor

20µl bacterial solution expressing recombinant ggSRBI-Fwd1-*ncol*/Rev1-*EcoRI*, used for immunization of rabbit 75/07, were used as competitor.

Surprisingly addition of recombinant peptide, used for immunization, to *rb-anti-ggSRBI* resulted in an increase in laying hen chicken liver total protein extract specific ggSRBI Western blot signal intensity. Pre-immune serum activity was not influenced by the competitor at all.

Results

As these results indicated a specific binding of rb-anti-ggSRBI to ggSRBI, the next question of interest was if rb-anti-ggSRBI would be functional for other species too. Therefore further Western blot experiments using rb-anti-ggSRBI as primary antibody, were performed on laying hen, mouse and quail liver total protein extracts. These type of Western blots were named zoo blot (Fig. 3.3.9) and performed under reducing (mercaptoethanol) and non-reducing conditions.

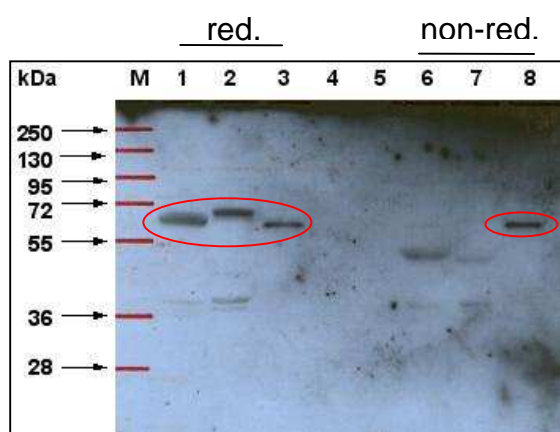


Fig.3.3.9

Zoo blot with rb-anti-ggSRBI

45µg of total liver protein extract per lane

1,6...Laying hen chicken

2,7...Quail

3,8...Mouse

4,5...Empty

Under reducing conditions distinct signals corresponding to similar molecular weights were detected for all three species. The molecular weight these bands corresponded to was ~64kDa for laying hen, ~67kDa for quail, and ~61kDa for mouse. Under non-reducing conditions the signals in quail liver and laying hen liver almost disappeared, whereas the intensity of mouse signal was not altered. These results possibly indicate a binding specificity of rb-anti-ggSRBI for SRBI in a broad variety of species.

3.4 Isoformsearch for ggSRBI

Before this work, nothing was known about SRB isoform SRBII in chicken. Both isoforms have been identified in other species like humans, cows, pigs, rats or mice. Thus, an investigation of the chicken genome for that isoform seemed worthy. Therefore a nucleotide alignment (Fig.3.4.1) of already known SRBII cDNA sequences was performed for identification of homology domains in the alternative exon of SRBII. These homology domains then should be used as target sequences for the reverse primer. Forward primer was set upstream of exon 13, where SRBI and SRBII sequences are identical (Fig.3.4.2).

Fig. 3.4.1 Nucleotide alignment of mouse, rat, human and bovine *SRBII* cDNA. Primer *ggSRBII-Rev1*, positioned in a highly homologous domain of these orthologs, is marked in red. The beginning of the alternative exon of *SRBII* is marked in blue.

Fig. 3.4.2 Nucleotide sequence of ggSRBI. Primer *ggSRBI-Fwd 1* is marked in red and the *last exon of ggSRBI* is marked in blue.

PCR Program

1 2

Bp M 1 2 3 4 5 6

500
400
300
200
100

The image shows an agarose gel electrophoresis result. On the left, a DNA ladder (Lane M) is visible with markers at 500, 400, 300, 200, and 100 bp. The gel has six lanes labeled 1 through 6. Lanes 1, 2, and 3 show no bands. Lane 4 shows a single band circled in red, which is approximately 200 bp in size. Lanes 5 and 6 show multiple bands, likely representing non-specific amplification or primer dimers.

Fig. 3.4.3

RT-PCR of laying hen chicken liver total RNA, using primer set ggSRBII-Fwd1/Rev1.

2,5...Liver total RNA

3,4...Negative control

Fig. 3.4.4 *EcoRI* restriction enzyme digestion of RT-PCR product *ggSRBII-Fwd1/Rev1* cloned into pCR 2.1-Topo. 1,2,3,4...Clones 1-4

Results

ggSRBI	GGGAAGATTGAGCCTGTGGTGATGCCGCTGCTGTGGTTTGCAGAGAGTGGATACATTGAT	1260
ggSRBII-Fwd1/Rev1	-----GAT	3

ggSRBI	GGCTCAGTCCTATCGCAGTTTTACACCAACCTGGTGCTGATCCCATCCATCCTAGACTAC	1320
ggSRBII-Fwd1/Rev1	GGCTCAGTCCTATCGCAGTTTTACACCAACCTGGTGCTGATCCCATCCATCCTAGACTAC	63

ggSRBI	CTGCAGTACATCTTCCTCGGGCTCAGCGTCCCGCTCATCATCTCAGCTGCACTCGTGATG	1380
ggSRBII-Fwd1/Rev1	CTGCAGTACATCTTCCTCGGGCTCAGCGTCCCGCTCATCATCTCAGCTGCACTCGTGATG	123

ggSRBI	ATGAGTCAGAAAGTCCTCAAGGAGAAGCACCCACACGACCCACACGAGAGCCCCCA	1440
ggSRBII-Fwd1/Rev1	ATGAGTCAGAAAGTCCTCAAGGAGAAGCACCCACACGACCCACACGAGAGCCCCCA	183

ggSRBI	CCCCAGCCCTCCGAGACCACACCGCTCCTCCAGGACACCGGCACACAGAGCCAGGACACC	1500
ggSRBII-Fwd1/Rev1	CCCCAGCCCTCCGAGACCACACCGCTCCTCCAGGACACCGGCACACAGAGCCAGGACACC	215

ggSRBI	GCCGAGGCCTGA	1512
ggSRBII-Fwd1/Rev1	-----	

Fig. 3.4.5 Nucleotide alignment of ggSRBI and PCR product of primer set **ggSRBII-Fwd1/Rev1** (marked in red). The last exon of ggSRBI is marked in blue.

Surprisingly, primer set ggSRBII-Fwd1/Rev1 seemed to amplify ggSRBI, even though ggSRBII-Rev1 was derived from a homology domain of SRBII cDNA sequences of different species. For further investigation of this phenomenon, amino acid sequence alignments of SRBI (Fig. 3.4.6) and SRBII (Fig. 3.4.7) of different species, with ggSRBI, were done.

mmSRBI	AMGGKPLSTFYTQLVLMQVVLHYAQYVLLGLGGLLLLVP IICQLRSQEKCFLEWWSGSKKG	480
rnSRBI	MMGGKTLNTFYTQLVLMQVVLHYAQYVLLGLGGLLLLVP I IYQLRSQEKCFLEWWSGSKKG	480
hsSRBI	AMEGETLHTFYTQLVLMQVLMHYAQYVLLALGCVLLLVPVICQIRSQEKCYLFWSSSKKG	480
ocSRBI	AMEGETLSTFYTQLVLLPNVLQYVLLALGCVLLLAPVIYQVRSQEKCYLFWSSSKKG	480
btSRBI	AMEGETLETFYIQLVLMQVVLHYAQYVLLALGCVLLL IPIYQIRSQEKCYLFWISFKKG	480
ggSRBI	YIDGSVLSQFYTNLVLIPSILDYLYIFLGLS-VPLIISAALVMMSQKSKEKHPDPTT	476
	: * . * ** :***:*.::* **::*.*. : *: . : **::.	
mmSRBI	SQDKEAIQAYSESLMSPAAGKTVLQEAKL	509
rnSRBI	SQDKEAMQAYSESLMSPAAGKTVLQEAKL	509
hsSRBI	SKDKEAIQAYSESLMTSAPKGSVLQEAKL	509
ocSRBI	SKDKEAAQAYSESLMTPDPKGTVLQEAKL	509
btSRBI	SKDKEAVQAYSEFLMTSAPKGTVLQEAKL	509
ggSRBI	QSPPPQFSETPLLQDTGTQSQDTAEA--	503
	.. . : * .. **	

Fig. 3.4.6 Amino acid sequence alignment of mouse, rat, human, rabbit, bovine, and chicken SRBI. A **homology domain** found in SRBI proteins of all species aligned, except ggSRBI, is marked in red. The **beginning of the last exon of SRBI** is marked in blue.

mmSRBII	AMGGKPLSTFYTQLVLMPOVLHYAQYVLLGLGGLLLLVPPIICQLRSQ	480
rnSRBII	MMGGKTLNTFYTQLVLMPOVLHYAQYVLLGLGGLLLLVPPIIYQLRSQ	480
hsSRBII	AMEGETLHTFYTQLVLMPOVMHYAQYVLLALGCVLLLPVICQIRSQ	480
ocSRBII	AMEGETLSTFYTQLVLLPNVLQYAYVLLALGCVLLLPVVIYQVRSQ	475
btSRBII	AMEGETLETFTYIQLVLMPOVLHYAQYVLLALGCVLLLPVVIYQIRSQ	480
ggSRBI	YIDGSVLSQFYTNLVLIPSILDYLYIFLGLSVPLIISAALVMMSQ	477
	: * . * ** :***:*.:. * **::*. * . *:: . : : .:	
mmSRBII	WSDQPPSPYTPILLEDLSGQPTSAMA	506
rnSRBII	WSDQPPSPYTPILLEDLSGQPASATS	506
hsSRBII	GPDRPPSPYTPLLPDSPSGQPPSPTA	506
ocSRBII	GPEQPAGPHTPLLQDSPCGPPAGPTA	501
btSRBII	QPDQLSPYTPLLQDSL RGQPTSPKV	506
ggSRBI	SPPPQSETTPLLDGTGTSQDTAEA	503
	. . . **** *:	

Fig. 3.4.7 Amino acid sequence alignment of mouse, rat, human, rabbit and bovine SRBII, and ggSRBI. A **homology domain** found in SRBII proteins of all species, but also in ggSRBI, is marked in red. The **beginning of the last exon of SRBII** is marked in blue.

The homology domain found in the last exon of those other species and chicken, was then used to design a new reverse primer, called ggSRBII-Rev3 (Fig. 3.4.8). For introduction of mouse liver total RNA as a positive control, a new forward primer called ggSRBII-Fwd3 (Fig. 3.4.9), located in a homology domain of mouse and chicken SRBI, was designed. Thus, this primer set was supposed to amplify mmSRBII and ggSRBII.

mmSRBII	ATCTGCCAACTGCGCAGCCAG	1440
rnSRBII	ATTTACCAACTGCGCAGCCAG	1440
hsSRBII	ATCTGCCAAATCCGAGCCAA	1440
btSRBII	ATCTACCAAATCCGAGCCAA	1440
	** * **** * ** ***** ** * ** * **** ** *** **	
mmSRBII	TGGTCAGACCAGCCACCCAGTCC	1500
rnSRBII	TGGTCAGACCAGCCGCCAGTCC	1500
hsSRBII	GGGCTGACCGGCCCCCAGCCC	1500
btSRBII	CAGCCAGACCAACTTCCCAGCCC	1500
	* * **** * ***** ***** ***** * * * *****	
mmSRBII	CCCACCAAGTGCCATGGCCTGA	1521
rnSRBII	CCCGCCAGTGCCACGTCCTGA	1521
hsSRBII	CCCCCAGCCCCACAGCCTGA	1521
btSRBII	CCCACCAAGTCCCAAAGTCTGA	1521
	*** **** ** *	

Fig. 3.4.8 Nucleotide alignment of mouse, rat, human and bovine SRBII. Reverse primer **ggSRBII-Rev3** is marked in red. The **beginning of the last exon of SRBII** is marked in blue.

Results

mmSRBI	TCTGCCCATGCCGAGAGTCTGGCATTGAGAATGTCAGCACCTGCAGGTTTGGTGCGCCTC	1018
ggSRBI	TCTGTCCCTGCAGGCAATCCGGCATCCAGAACGTCAGCTCCTGCAGGCTGAATGCACCAA	1009
	***** ** *	
mmSRBI	TGTTTCTCTCCACCCCACTTTTACAACGCCGACCCTGTGTTGTCAGAAGCTGTTCTTG	1078
ggSRBI	TGTTTCATCTCCACCCCTCACCTTCTACAATGCTGACCCAAGCCTGGTGGATGCTGTGAAG	1069
	***** ** *	
mmSRBI	GTCTGAACCCTAACCCAAAGGAGCATTCCTTGTTCCTAGACATCCATCCGGTCACTGGGA	1138
ggSRBI	GCCTGCACCCAGCAGGGAGGAGCACGCGCTCTTCCTCGATGTGCACCCGGTGACAGGCA	1129
	* *	
mmSRBI	TCCCCATGAACTGTTCTGTGAAGATGCAGCTGAGCCTCTACATCAAATCTGTCAAGGGCA	1198
ggSRBI	TCCCCATGAACTGCTCCATCAAGCTCCAGCTGAACCAGTACATGAAGCAGGTGTCTGGTA	1189
	***** *	
mmSRBI	TCGGGCAAACAGGGAAGATCGAGCCAGTAGTTCTGCCGTTGCTGTGGTTTGAACAGAGCG	1258
ggSRBI	TCCTTCAAACAGGGAAGATTCAAGCTGTGGTGATGCCGCTGCTGTGGTTTGCAGAGAGTG	1249
	** ***** *	
mmSRBI	GAGCAATGGGTGGCAAGCCCCTGAGCACGTTCTACACGCAGCTGGTGCTGATGCCCCAGG	1318
ggSRBI	GATACATTGATGGCTCAGTCCTATCGCAGTTTACACCAACCTGGTGCTGATCCCACATCA	1309
	** *	
mmSRBI	TTCTTCACTACGCGCAGTATGTGCTGCTGGGGCTTGAGGGCTCCTGTTGCTGGTGCCCA	1378
ggSRBI	TCCTAGACTACCTGCAGTACATCTTCCTCGGGCTCAGCGTCCCGCTCAT-----CATC	1362
	* *	
mmSRBI	TCATCTGCCAACTGCGCAGCCAGGAGAAATGCTTTTGTGTTTGGAGTGGTAGTAAAAAGG	1438
ggSRBI	TCAGCTGC-ACTCGTGATGATGAGTCAGAAAGTCCTCCA--AGGAGAAGCACCACACGA	1418
	*** *	

Fig. 3.4.9 Nucleotide alignment of mouse and chicken SRBI. Forward primer ggSRBII-Fwd3 is marked in red. The beginning of the last exon of SRBI is marked in blue.

Primer set ggSRBI was used for RT-PCR (Fig. 3.4.10) of laying hen and mouse liver total RNA, resulting in PCR products of 405bp length, which were then cloned into pCR 2.1-Topo (Fig. 3.4.11) for sequencing (3.4.12).

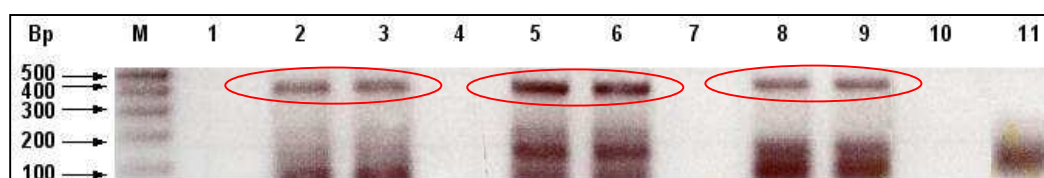


Fig. 3.4.10 RT-PCR of laying hen and mouse liver total RNA, using primer set ggSRBII-Fwd3/Rev 3.

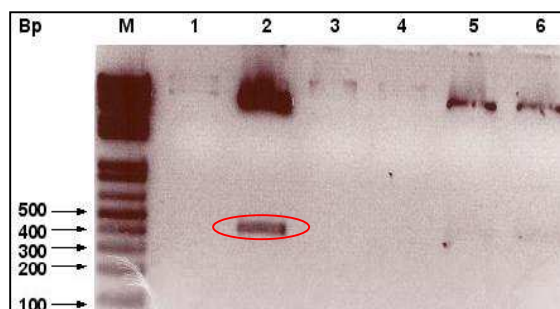
2,3,...Chicken total RNA 1

5,6...Chicken total RNA 2

8,9...Mouse total RNA

11...Negative control

1,4,7,10...Empty



EcoRI restriction enzyme digestion of
ggSRBII-Fwd3/Rev3 PCR product cloned
into pCR 2.1-Topo.
1.2.3.4.5.6... Clones 1-6

Positive clone 2 was sequenced and a nucleotide alignment of ggSRBII-Fwd3/Rev3 PCR product and ggSRBI was done. GgSRBII-Fwd3 was localized in a region identical for SRBI and SRBII, way upstream of the alternative exon, from nucleotide 1010-1029. Therefore, if these primers amplified ggSRBII, 380 nucleotides of the derived DNA fragment should match SRBI perfectly.

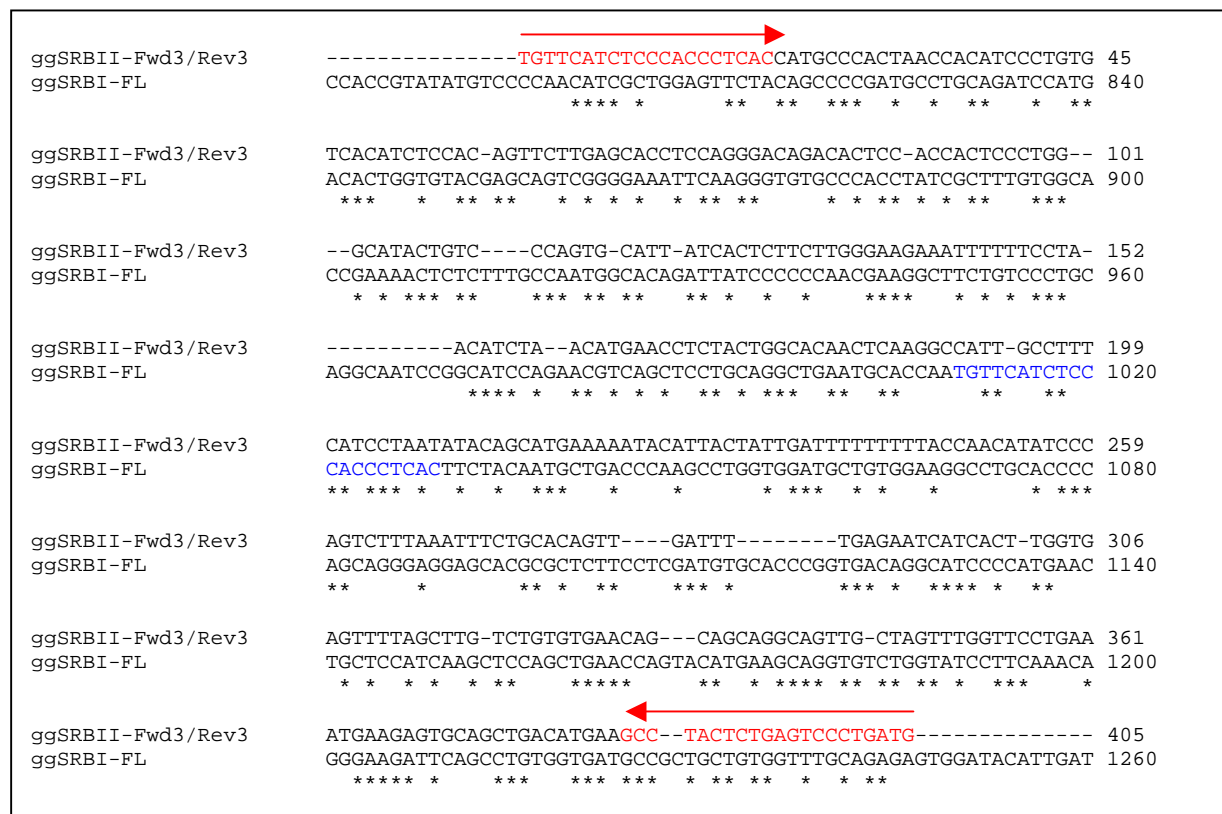


Fig. 3.4.12 Nucleotide alignment of ggSRBI and PCR product of primer set ggSRBI-Fwd3/Rev3 (marked in red). Position of ggSRBI-Fwd3 sequence in ggSRBI is marked in blue.

Results

The DNA fragment derived from primer set ggSRBI-Fw3/Rev3 did not match SRBI, indicating unspecific amplification of DNA fragments.

After those negative results so far, one last PCR approach was performed, using ggSRBII-Fwd 1.2/Rev3 as primer set (Fig. 3.4.13). GgSRBII-Fwd1.2 corresponded to the same position as ggSRBII-Fwd1, but its 3' end was extended for 3 nucleotides, so PCR conditions of the forward and reverse primer were more similar. The PCR product of this primer set therefore should be smaller, than that of ggSRBII-Fwd3/Rev3, increasing the possibility of successful PCR.

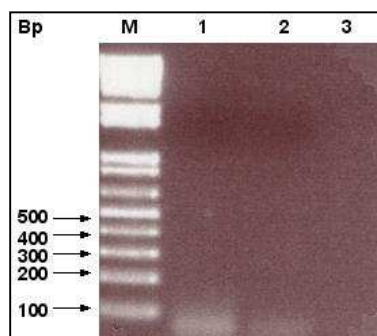


Fig. 3.4.13

RT-PCR of laying hen liver total RNA, using ggSRBII-Fwd1.2/Rev3 as primer set.

1...Laying hen

2...Negative control

3...Empty

Though using different PCR programs, no product could be obtained, probably indicating problems in reverse primer binding to the homology domain, which the reverse primer was located to. PCR mediated amplification of SRBII also might have failed, because that SRB isoform does not exist in chicken.

4 Discussion

Human Scavenger Receptor Class B Type I (SRBI) has been identified as the first physiologically relevant HDL-binding receptor. Its main function is mediating selective lipid uptake. Due to its role in HDL metabolism and its expression in many different tissues and cell types, SRBI affects several physiological processes of great medical interest, such as atherosclerosis, female fertility, fetal development and others mentioned in the Introduction. Chicken is a well characterized and easy to manipulate model organism for lipid metabolism. Thus, this diploma work was performed to identify and characterize putative chicken SRBI (ggSRBI) and its possibly existing isoform, SRBII.

Chicken ENSEMBL database search revealed an mRNA sequence (GENSCAN00000043265) referred to as ggSRBI. According to this sequence, primers were designed and used for amplification of full-length ggSRBI cDNA from laying hen liver total RNA by RT-PCR. The resulting PCR product was then cloned into apCR 2.1-TOPO plasmid and sequenced. The amplified PCR product was 100% identical with the sequence derived from ENSEMBL database. The open reading frame coding for ggSRBI is of a length of 1512bp and is translated to a 503 amino acid polypeptide with a predicted molecular weight of ~56kDa. Human SRBI is heavily glycosylated and palmitoylated, and thus its apparent molecular weight is 82kDa, significantly larger than the predicted size of 57kDa.. It is not yet known whether ggSRBI is similarly glycosylate.

For further investigations of ggSRBI at the protein level, an anti-ggSRBI antibody was generated. A fragment of ggSRBI, from amino acids 42-276, was recombinantly expressed as HIS-tag, HSV-tag fusion protein of a predicted molecular weight of ~31kDa in E.coli. Purification of the recombinant protein using Ni-NTA agarose was unfortunately unsuccessful. Once the recombinant protein had bound to the Ni-NTA agarose, it did not dissociate again, even though several modified approaches were performed. Thus, the recombinant protein was separated by performing polyacrylamide gel electrophoresis, visualized by Coomassie blue staining and

excised directly from the gel. A rabbit was immunized with the recombinant protein and the immune serum was tested for its ability to recognize ggSRBI.

Western blots of laying hen liver total protein extracts were performed, using the rabbit serum as primary antibody and pre-immune serum from the same rabbit as negative control. First, different dilutions (1:100, 1:500, 1:1000) of immune serum in 5% milk, TBST, 2mM CaCl₂ (blocking solution) were tested. All 3 dilutions resulted in visualization of a band in laying hen liver total protein extracts of ~64kDa, and according to the dilution of the immune serum the signal varied in intensity. Furthermore, this band was not obtained using pre-immune serum as primary antibody, thus indicating specific reactivity. The predicted molecular weight of ggSRBI is 56kDa as mentioned above, but if ggSRBI is glycosylated like human SRBI, the obtained 64kDa signal in Western blotting might correspond to ggSRBI. A dilution ratio of immune serum and blocking solution of 1:500 is quite useful, as a distinct signal could be obtained and anti-serum could be saved. Thus, this dilution was used for further Western blots using the antiserum as primary antibody.

Denaturation of proteins during SDS-PAGE under reducing conditions may influence the binding ability of the antibody used for its detection. Thus, Western blots of laying hen liver total protein extracts were performed under reducing and non-reducing conditions. Under reducing conditions, a signal was obtained similar to that described above, whereas under non-reducing condition the 64kDa-band almost disappeared. Instead, other bands of 50kDa, 90kDa, 95kDa and higher became visible. As human SRBI is known to dimerize, this could be true for ggSRBI as well, and thus the 90kDa and 95kDa signals may be due to such homodimerization. As mentioned, the 50 kDa signal was the most intensive one, but this band was also obtained using pre-immune serum, indicating unspecific antibody binding. Most probably the antiserum binds non-reduced ggSRBI less efficiently, maybe due to epitope inaccessibility. For further investigation of ggSRBI binding specificity of the immune serum, competition assays were performed of laying hen total protein extracts by including the recombinantly expressed protein used for immunization as competitor for the antigen-antibody interaction.. Immune serum was supposed to bind to the added recombinant protein, thereby limiting ggSRBI specific antibodies in the serum available for binding to the ggSRB in the protein extract. Surprisingly, addition of the competitor increased the intensity of the 64kDa signal obtained, instead of decreasing it as supposed. Dimerization of ggSRBI bound to the nitrocellulose

membrane with the recombinantly expressed protein added to the incubation mixture might be the cause for this phenomenon. As mentioned above, human SRBI is known to dimerize, therefore dimerization of ggSRBI and the recombinantly expressed fragment of ggSRBI is quite possible, and thus it can be concluded that the antibodies indeed bind to ggSRBI in a specific manner.

Binding specificity of rb-anti-ggSRBI was tested also in other species. Therefore a Western blot of liver total protein extracts from laying hen, mouse and quail was performed using the antiserum as source of primary antibodies. Distinct signals were obtained for all three species: the usual 64kDa signal in laying hen, a 61kDa signal in mouse, and a 67kDa signal in quail. No other signals of significant intensity were obtained, again suggesting specific binding of rb-anti-ggSRBI to quail and mouse SRBI. This antiserum therefore might be a useful tool for further investigations of SRBI in species other than chicken as well.

SRBI is expressed in many different cell types and tissues, and at different levels. Gene expression of SRBI was investigated in this diploma work, performing Northern blot analysis of total RNA isolated from different tissues. For optimization of the Northern blot, only tissues known to express SRBI at high levels in other species were used, namely, liver and adrenals. GgSRBI fragments from nucleotide 124-828 and 93-1422, as well as an ApolipoproteinAV probe kindly donated by Dr. Andrea Dichlberger, were used as radiolabelled probes. ApoAV mRNA, present in liver extracts, served as positive control. Northern blotting of liver and adrenal total RNA extracts was performed and probes were added, but the only signal obtained was from the ApoAV-probe in liver total RNA extract. A double band at 2.3kb and ~1.4kb was obtained, consistent with former findings of Dr. Andrea Dichlberger, indicating functional Northern blotting. According to the ENSEMBL database, ggSRBI mRNA consists of 1821bp. Yet, no signal was obtained from either of the two ggSRBI probes used. GgSRBI probe 93-1422 even seemed to stick to the markers, a phenomenon not observed with the ggSRBI probe 124-828. Lowering the incubation temperature for ggSRBI probe 124-828 to make Northern blot conditions less stringent, did also not result in a ggSRBI signal. Probes were labelled according to the manufacturer's protocol and fractionated with NICK columns. Radioactive nucleotide incorporation was verified using a Geiger counter. Additionally, the positive control was positive,

Discussion

indicating successful Northern blotting. Thus, the ggSRBI fragments used or the preparation of the mRNA may have been the cause of the problem. Probably, secondary structures within either the probes or the mRNA impaired probe binding. Possibly, real-time PCR techniques may solve the problems and allow investigation of ggSRBI mRNA levels.

Alternatively spliced variants of SRBI are known to exist in humans and many other species. So-called SRBII has been described for human, cow, pig, rat, and mouse. Thus, an isoform-search was performed using RT-PCR to amplify ggSRBII from laying hen liver total RNA. In all species studied to date, SRBI and SRBII only differ in their last exon. This last exon codes for the C-terminal cytoplasmatic domain of the protein, which is thought to mediate cellular responses to receptor binding. Due to that essential function, a conserved region, within the last exon, typical for one or the other isoform was expected to exist. Thus, mouse, human, cow, and rat SRBII cDNA nucleotide sequences were aligned and searched for a highly conserved region in the last alternative exon of SRBII. Forward PCR primers were set upstream, where SRBI and SRBII nucleotide sequences are identical, and reverse primers were set in the conserved region within the last SRBII exon. The PCR product then was cloned into a pCR 2.1-TOPO vector, amplified in *E.coli* and sequenced. Surprisingly, the amplified part was 100% identical with the putative ggSRBI, even though the reverse primer was designed according to SRBII sequences of other species. Thus, mouse, rat, human, cow, and rabbit SRBI and SRBII protein amino acid sequences were aligned and compared to ggSRBI. SRBI proteins from all species mentioned share an SESL motif within their last exon, and SRBII proteins of all species mentioned share a TPLL motif within their last exon. In contrast, the obtained SRB from chicken contained the otherwise typical SRBII motif TPLL, indicating that the protein referred to as ggSRBI, should in fact be considered to be ggSRBII. Therefore, I designed new primers targeted to the SRBI-typical SESL motif found within the last exon and a region upstream of the last exon, conserved in ggSRBI and mmSRBI. This new primer set was supposed to amplify mmSRBI as positive control, and potentially also ggSRBI (and not the protein named ggSRBI so far). PCR products of ~400bp length were obtained by RT-PCR of laying hen and mouse liver total RNA. PCR products were cloned into pCR 2.1-TOPO and transformed into *E.coli*, but only colonies of bacteria containing the fragment amplified from the

chicken transcripts could be obtained. Even though PCR of mouse liver total RNA was repeated, it was not possible to obtain any transformed bacterial cells. Since the forward primer for the PCR was set within the area shared by SRBI and SRBII, if amplification of the potentially new ggSRBI was successful, 380 nucleotides were expected to be identical with the mRNA sequence referred to as “ggSRBI” so far. Yet, sequence analysis of the amplified fragment revealed no sequence homology between the fragment and ggSRBI sequence at all. Thus, in this diploma work it was not possible to identify a second alternatively spliced isoform of the protein referred to as ggSRBI in this Thesis.

Taken together, these findings strongly suggest that, based on the presence of the otherwise typical SRBII-motif TPLL found in the chicken sequence, that the characterized cDNA is in fact encoding chicken scavenger receptor class B type II (ggSRBII), and not ggSRBI. Future searches on SRB isoforms - in chicken should focus on SRBI or on other not yet known types of alternative splice variants. Following the suggestion of renaming ggSRBI to ggSRBII, ggSRBII would share the typical TPLL motif found in SRBII of other species. Therefore it seems likely that a type I isoform in chicken should contain the SESL motif of SRBI found in other species. Still, it was not possible to amplify from total RNA an isoform containing the homology region of SRBI proteins of other species- Possibly, primers set in the homology region could not bind due to steric reasons like secondary structures, or a type-I isoform of SRB in chicken actually does not exist. It should be noted that the Western blots showed only one band, despite the fact that the antibody raised should react with the hypothetical SRBI as well; however, if the chicken SRBs were splice variants analogous to those in mammals, their size difference might be too small to resolve two bands. Future studies will address this point as well.

5 References

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6 Abstract

Besides its housekeeping functions in all mammalian cells, cholesterol contributes to different biological functions like bile acid-, vitamin D- or steroid hormone synthesis. Thus, multiple pathways have evolved to ensure full supply of animal cells with cholesterol, involving different lipoproteins and cell surface receptors.

Human SRBI, initially identified in 1994 by Endemann, Acton, Krieger and colleagues, was the first HDL receptor of physiological relevance to be found. SRBI is a member of a family of structurally related proteins, called the CD36 superfamily. It is anchored in the cellular plasma membrane and found in cells of a wide variety of tissues, where expression levels are highest in liver and steroidogenic tissues. The main function of SRBI is mediating bi-directional selective lipid transfer, mainly in the form of cholesteryl esters, from HDL particles to and from cells. Hepatic overexpression of SRBI in mice leads to decreased plasma HDL cholesterol concentration, as well as to an increase in hepatic bile cholesterol concentration, whereas SRBI knock-out mice show a 2-2.5-fold elevated total plasma cholesterol concentration, caused by an increase in large HDL particles heterogeneous in size, which are abnormally enriched in apoprotein E. These findings indicated that SRBI is important for cellular cholesterol uptake from HDL particles, thereby ensuring sufficient cholesterol supply of steroidogenic tissues, as well as reverse cholesterol transport from extrahepatic tissues to the liver, thus mediating secretion of excess cholesterol into bile. Due to the reported physiological functions, SRBI is supposed to be involved in different pathophysiological processes like atherosclerosis or female infertility. Thus, SRBI is a promising target for investigation of the molecular mechanisms underlying these pathological conditions.

Most of the studies concerning SRBI were performed using rodents as model organisms. Yet, as chicken is a well characterized and easy to manipulate model organism for studies on lipid metabolism, this diploma work was performed to identify and characterize possibly existing chicken SRBI (ggSRBI). Therefore, ggSRBI was cloned from laying hen chicken liver total RNA extracts and an antibody was raised against it, using a recombinantly expressed fragment of ggSRBI for immunization of a rabbit. Performing Western blot experiments, resulting in a specific ~64kDa signal, binding specificity of the antibody was proven. Furthermore, gene expression of SRBI

in different tissues was investigated, performing Northern blotting of laying hen total tissue RNA extracts. However, although using different radiolabelled probes and experimental conditions, no ggSRBI mRNA signal could be obtained. An isoform search for SRBI was performed, as in other species like human, mouse or rat, an alternatively spliced isoform of SRBI, called SRBII, had been found. Therefore RT-PCR of laying hen liver total RNA using forward primers set in a region identical for both isoforms, and reverse primers set in a conserved homology region of known SRBII sequences of other species, was performed. Although using different PCR programs and primer sets, no isoform could be obtained. Yet, it could be shown that the mRNA sequence referred to as SRBI, derived from ENSEMBL chicken database, surprisingly contained a homology motif, which was found in SRBII-, but not in SRBI-sequences of other species. This indicates that the cDNA characterized here in fact is encoding ggSRBII rather than ggSRBI.

7 Zusammenfassung

Neben seiner Haushaltsfunktionen in allen Säugetierzellen, trägt Cholesterin zu verschiedenen biologischen Funktionen bei, wie zum Beispiel zu Gallensäureproduktion, Vitamin D- und Steroidhormon- Synthese. Verschiedene Wege zur Gewährleistung der vollen Versorgung der tierischen Zellen mit Cholesterin sind evolutionär entstanden, inklusive verschiedener Lipoproteine und Zelloberflächenrezeptoren. Humanes SRBI, 1994 erstmals von Endemann, Acton, Krieger und Kollegen identifiziert, war der erste physiologisch relevante HDL Rezeptor der gefunden wurde. SRBI ist Mitglied einer Familie von strukturverwandten Proteinen, der CD 36 Superfamilie. Es ist in der zellulären Plasmamembran verankert und in einer großen Anzahl an verschiedenen Geweben gefunden worden, obgleich der Expressionslevel in Leber und steroidogenen Geweben am höchsten ist. Die Hauptfunktion von SRBI ist die Vermittlung von bidirektionalem selektivem Lipidtransfer, hauptsächlich in Form von Cholesterylestern, von HDL Partikeln zu Zellen und umgekehrt. Hepatische Überexpression von SRBI in Mäusen führt zu einer Senkung der Plasma HDL-Cholesterinkonzentration, während SRBI knockout-Mäuse einen 2-2,5 fachen Anstieg der totalen Plasmacholesterinkonzentration zeigen, verursacht durch einen Anstieg an großen HDL Partikeln, welche heterogen in ihrem Umfang und außergewöhnlich mit apolipoprotein E angereichert sind. Diese Ergebnisse deuten darauf hin, dass SRBI für die zelluläre Aufnahme von Cholesterin wichtig ist, wodurch die ausreichende Versorgung von steroidogenen Geweben mit Cholesterol gewährleistet wird, sowie den entgegengesetzten Cholesteroltransport von extrahepatischen Geweben zur Leber, wodurch die Sekretion von überschüssigem Cholesterin in die Gallensäure vermittelt wird. Aufgrund der festgestellten physiologischen Funktionen wird vermutet, dass SRBI in verschiedene pathophysiologische Prozesse, wie Atherosklerose und weibliche Unfruchtbarkeit, involviert ist. Daher ist SRBI ein vielversprechendes Ziel zur Untersuchung der molekularen Mechanismen, die diesen pathologischen Bedingungen zu Grunde liegen. Die meisten Studien betreffend SRBI wurden in Nagetieren als Modellorganismen durchgeführt. Da jedoch das Haushuhn ein gut charakterisierter und leicht zu manipulierender Modellorganismus für das Studium des Fettstoffwechsels ist, wurde

diese Diplomarbeit durchgeführt, um möglicherweise existierendes Hühner SRBI (ggSRBI) zu identifizieren und zu charakterisieren. Dafür wurde ggSRBI aus Total RNA-Extrakten einer Legehennenleber kloniert und ein Antikörper dagegen erzeugt, indem ein rekombinant exprimiertes Fragment von ggSRBI verwendet wurde, um ein Kaninchen zu immunisieren. Western blot Experimente, welche ein ~64kDa Signal ergaben, wurden durchgeführt, um die Bindungsspezifität des Antikörpers zu beweisen. Des weiteren wurde die Genexpression von ggSRBI in verschiedenen Geweben mittels Northern blotting von Total RNA-Extrakten einer Legehennenleber untersucht. Aber obwohl verschiedene radioaktive Sonden und unterschiedliche experimentelle Bedingungen verwendet wurden, konnte kein mRNA Signal erhalten werden. Eine Isoformsuche für ggSRBI wurde durchgeführt, da in anderen Spezies wie Mensch, Maus und Ratte eine alternativ gespleißte Isoform von SRBI namens SRBII gefunden wurde. Dafür wurden RT-PCRs mit RNA aus Legehennenleber durchgeführt, unter Verwendung von Vorwärts-Primern, die für eine Region in der die beiden Isoformen identisch sind bestimmt waren, und mit Rückwärts-Primern, korrespondierend mit einer konservierten homologen Region der bekannten SRBII Sequenzen anderer Spezies. Obwohl verschiedene PCR Programme und Primer verwendet wurden, konnte keine Isoform entdeckt werden. Es war jedoch möglich zu zeigen, dass die mRNA Sequenz, die bisher als SRBI bezeichnet wurde, und von ENSEMBL stammte, überraschenderweise ein homologes Motiv beinhaltete, welches zwar in SRBII, aber nicht in SRBI Sequenzen von anderen Spezies vorkommt. Dies deutet darauf hin, dass die cDNA, die in dieser Diplomarbeit charakterisiert wurde, tatsächlich für ggSRBII und nicht für ggSRBI kodiert.

8 Lebenslauf

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Besondere Kenntnisse

Sprachkenntnisse	Deutsch: Muttersprache Englisch: Fließend
EDV-Kenntnisse	MS Word, MS Photo Editor, MS Powerpoint, Online Programme zum molekularbiologischen arbeiten: Fortgeschritten MS Excel: Grundlegend
Wissenschaftliche Fähigkeiten	Fortgeschrittene Fähigkeiten zur selbständigen wissenschaftlichen Projekt- und Versuchsplanung. Fortgeschrittene Fähigkeiten zur selbständigen Anwendung von zellbiologischen-, mikrobiologischen- und biochemischen Methoden, sowie zur Auswertung und Interpretation der erhaltenen Daten